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Applicant: Ray W. Wood et al.

Title: METHODS OF ADMINISTERING LIQUID DROPLET AEROSOLS OF NANOPARTICULATE DRUGS

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DECLARATION UNDER 37 C.F.R. §1.132

The undersigned, Gary G. Liversidge, hereby declares as follows:

I. Background of Gary G. Liversidge

1. I received my Ph.D. in 1981 from the University of Nottingham, England, in Pharmaceutical Chemistry. I have been working in the field of nanoparticulate drug technology since 1987, when I joined Eastman Pharmaceuticals.

2. Through a series of business transactions, Eastman Pharmaceuticals became Sterling Winthrop Pharmaceuticals Research Division, and then NanoSystems. This business then became known as the Elan Drug Technologies (EDT) business division of Elan Corp. PLC. EDT merged with Alkermes, Inc. on September 8, 2011 to form Alkermes, plc. Through a series of business transactions the current owner of the present application is Alkermes Pharma Ireland Limited.

3. Currently I am Chief Technology Officer and Vice President, R&D, at Alkermes plc, based at 852 Winter Street, Waltham, MA 02451.

II. Not all active agents can be formulated into stable nanoparticulate active agent compositions

A. It is unpredictable whether a functional equivalent can be successfully made into a nanoparticulate active agent composition.

4. It is unpredictable whether a functional equivalent of a compound can be successfully made into a nanoparticulate active agent composition. This fact is demonstrated by the data below, detailing the successful preparation of a stable nanoparticulate cilostazol composition, in contrast to the unsuccessful attempts to make a stable nanoparticulate clopidogrel bisulphate composition. Clopidogrel is an inhibitor of platelet aggregation, and is a functional equivalent of cilostazol in inhibiting platelet aggregation.

(1) Stable nanoparticulate cilostazol composition was successfully obtained.

5. Cilostazol is a drug used to inhibit platelet aggregation. *See* abstract by Kimura et al., *Arzneimittelforschung*, *35*(7A): 1144-1149 (1985) (Exhibit 1). Cilostazol is a poorly water soluble drug having an aqueous solubility of 3 μg/mL at 25°C or 6 μg/mL at 37°C.

6. As demonstrated by Jinno et al., *J. Controlled Release*, *111*: 56-64 (2006) (Exhibit 2), a stable nanoparticulate cilostazol composition was successfully obtained by Elan's (now Alkermes') NanoCrystal® technology. *See* page 57, right column, section 2.3 for "Preparation of cilostazol suspensions." The obtained nanoparticulate cilostazol composition comprised hydroxypropyl cellulose and docusate sodium as the surface stabilizers. *Id.*, section 2.2 for "Particle size reduction of cilostazol." The nanoparticulate cilostazol composition had a median particle size of 0.22 μm (or 220 nm). *See* page 59, Figure 1, and section 3.1 for "Size distribution of milled cilostazol."

7. Exhibit 2 further demonstrates superior solubility, dissolution, bioavailability, and elimination of food effect for the nanoparticulate cilostazol composition in comparison to the

hammer-milled and jet-milled cilostazol compositions having a median particle size of 13 μm and 2.4 μm, respectively. *See* pages 59-63.

8. Accordingly, a nanoparticulate cilostazol composition was successfully obtained using NanoCrystal® technology, as demonstrated by the published scientific literature.

(2) Stable nanoparticulate clopidogrel bisulphate composition could not be obtained.

9. Clopidogrel is an inhibitor of platelet aggregation, and is a functional equivalent of cilostazol in inhibiting platelet aggregation. Clopidogrel bisulphate is insoluble in water at neutral pH but freely soluble in water at a pH of 1.0. Clopidogrel, as a methyl ester, is hydrolysed *in vivo* by esterases to an inactive carboxylic acid derivative, which represents more than 85% of the circulating drug related compounds in the plasma. Thus, only a small unknown portion of clopidogrel is available for metabolism to the active metabolite after oral administration. Therefore, it is highly desirable to obtain a stable nanoparticulate clopidogrel bisulphate composition to improve bioavailability of this drug.

10. The challenge in obtaining a stable nanoparticulate clopidogrel bisulphate composition is that during the milling process to reduce the particle size of clopidogrel, the pH of the milling mixture decreases while the dissolution of clopidogrel bisulphate increases. Accordingly, clopidogrel bisulphate undergoes auto-catalysis during the milling process until it is completely dissolved at pH 1-2. Solubilizing clopidogrel bisulphate does not solve the problem of poor bioavailability as *in vivo* the drug is hydrolysed by esterases to an inactive carboxylic acid derivative, as noted above.

11. Using the same NanoCrystal® technology, different approaches were attempted to stabilize particulate clopidogrel bisulphate during the milling process, such as milling in buffered systems and milling using the common ion effect, although none of these approaches resulted in a stable nanoparticulate clopidogrel bisulphate composition.

12. In a first set of experiments, milling of clopidogrel bisulphate was conducted in different buffered systems having a pH from 6.0 – 12.0 to prevent dissolution of clopidogrel bisulphate during milling. The results are detailed in the table below.

Table 1

Clopidogrel Bisulphate (% w/w)	Surface Stabilizer (% w/w)	Buffered System (pH)	pH prior to milling	pH post milling
5%	HPC-SL (2%)	93% w/w citric acid/sodium phosphate dibasic solution (pH 7.0)	not determined	2.21
5%	Plasdone S-630 (2%)	93% w/w sodium phosphate monobasic/sodium phosphate dibasic solution (pH 8.0)	7.0	1.82
5%	Plasdone K29/K32 (2%)	93% w/w hydrochloric acid-Tris (hydroxymethyl) amino methane solution (pH 8.9)	2.0	Not milled due to dissolution of active agent prior to milling
5%	HPC-SL (2%)	93% w/w citric acid/sodium phosphate dibasic solution (pH 7.0)	6.0	2.17
5%	HPC-SL (2%)	93% w/w buffered solution (pH 12.0)	3.0	Not milled due to dissolution of active agent prior to milling
5%	Pharmacoat 603 (2%)	93% w/w simulated intestinal fluid (pH 12.0)	Not determined	2.0

13. Due to the dissolution of clopidogrel bisulphate in the buffered systems either prior to or post milling, despite the variable conditions attempted, it was found that it was ***impossible*** to obtain a stable nanoparticulate clopidogrel bisulphate compositions.

14. In a second set of experiments, milling of clopidogrel bisulphate was conducted under the condition of saturating the system with common ion, bisulfate ion, in a saturated

sodium bisulphate solution to control the equilibrium solubility of the clopidogrel during milling. The results are detailed in the table below.

Table 2

Clopidogrel Bisulphate (% w/w)	Surface Stabilizer (% w/w)	Observations
5%	None	Microscopy showed the presence of drug particles in small quantities. The majority of material observed was in a flocculated state.
5%	Pharmacoat 603 (HPMC) (1%)	After subsequent addition of stabilizer, flocculation appeared to be reduced but still present. An increased proportion of the drug appeared in the harvested aliquot suggesting increased milling of the drug. Brownian motion was not apparent.
5%	HPC-SL (2%)	Some milled drug particles were apparent although in very small quantities. Particles did not exhibit Brownian motion. Flocculation was readily apparent throughout the aliquot of sample observed under microscope.
5%	Tween 80 (2%)	Unmilled drug particles apparent. Aliquot harvested for microscopy was extremely dilute suggesting that very little drug was milled.
5%	Pharmacoat 603 (HPMC) (2%)	Nanoparticles were observed in very small quantities. Particle did exhibit a small degree of Brownian motion.
5%	Pharmacoat 603 (HPMC) (2%), DOSS (0.05%)	Very small quantities of milled drug particles were observed. Although particles were somewhat milled, they did not appear to be in the nanoparticulate size range. No Brownian motion was observed
5%	Plasdone S-630 (2%)	Harvesting was not possible as the drug did not appear to mill. No microscopy was therefore performed. A large proportion of the slurry was observed on upper mill plate and agitator possibly leading to a void in the mill chamber reducing the milling efficiency.
5%	Tyloxapol (1%)	Microscopy showed the presence of a very small concentration of milled particulates which appeared to exhibit Brownian motion most likely due to the diluted nature of the slurry.

15. Despite the variable conditions attempted, it was found that it was ***impossible*** to obtain a stable nanoparticulate clopidogrel composition by controlling the equilibrium solubility of clopidogrel bisulphate via saturating the system with the common ion.

16. Accordingly, a stable nanoparticulate clopidogrel bisulphate composition could not be obtained using the NanoCrystal® technology under the various conditions tested.

B. Stable nanoparticulate orlistat compositions could not be obtained.

17. Orlistat is a drug for treating obesity by preventing the absorption of fats from diet, thereby reducing caloric intake. However, orlistat is associated with significant gastrointestinal side effects, including steatorrhea, fecal incontinence, and frequent or urgent bowel movements. Therefore, it is desirable to obtain a nanoparticulate orlistat composition to alleviate the side effects.

18. The challenge in obtaining a stable nanoparticulate orlistat composition is that despite the attempts to mill approximately 30 different orlistat formulations, it was very challenging to obtain a nanoparticulate orlistat composition having the desired particle size due to a number of problems, such as difficulty encountered to "wet in" orlistat for milling, a significant quantity of unmilled orlistat remaining in the milling chamber, and difficulty encountered to harvest the milled orlistat.

19. Even when a nanoparticulate orlistat composition having the desired particle size was initially obtained, the composition was unstable under common storage conditions for a period of 14 days, as detailed in the table below.

Table 3

Formulation	Storage Time (days)	Storage Condition	Mean (nm)	D50 (nm)	D90 (nm)	D95 (nm)
Orlistat, 5%w/w	0	N/A	436	339	658	1009
Pharmacoat 603, 2%w/w	0	N/A	390	334	599	795

Table 3						
Formulation	**Storage Time (days)**	**Storage Condition**	**Mean (nm)**	**D50 (nm)**	**D90 (nm)**	**D95 (nm)**
(Hydroxypropyl methylcellulose) Deionised Water, 93%w/w	**14**	**5°C**	**50732**	**23999**	**139683**	**178902**
	14	**5°C**	**2045**	**362**	**7376**	**13593**
Orlistat, 5%w/w Pharmacoat 603, 1.25%w/w (Hydroxypropyl methylcellulose) Lauryl Sulfate, 0.05%w/w (Sodium Lauryl Sulfate) Deionised Water, 93.7%w/w	0	N/A	196	185	258	295
	0	N/A	195	185	257	294
	14	**5°C**	**28076**	**196**	**100516**	**136455**
	14	**5°C**	**2439**	**208**	**8771**	**18960**
	14	**25°C/60% RH**	**16072**	**170**	**72678**	**104171**
	14	25°C/60% RH	1214	212	717	7838
Orlistat, 5%w/w Pluronic F108, 1.5%w/w (Poloxamer 338) Deionised Water, 93.5%w/w	0	N/A	649	296	1907	2925
	0	N/A	580	292	1487	2628
	14	**5°C**	**38717**	**15805**	**106358**	**133797**
	14	5°C	314	164	286	693
Orlistat, 5%w/w Pharmacoat 603, 1.25%w/w (Hydroxypropyl methylcellulose) Lauryl Sulfate, 0.05%w/w (Sodium Lauryl Sulfate) Deionised Water, 93.7%w/w	0	N/A	400	289	537	1017
	0	N/A	340	286	485	673
	14	**5°C**	**64545**	**4924**	**209398**	**266403**
	14	**5°C**	**3405**	**311**	**12049**	**24135**
	14	**25°C/60% RH**	**13556**	**189**	**58786**	**88122**
	14	25°C/60% RH	814	211	984	3870
Orlistat, 5%w/w Pluronic F108, 1.00%w/w (Poloxamer 338) Tween 80, 1.00%w/w (Polyoxyethylene Sorbitan Fatty Acid Esters) Deionised Water, 93%w/w	0	N/A	176	164	225	261
	0	N/A	168	161	219	246
	14	5°C	1790	152	486	8500
	14	5°C	219	194	272	345
	14	**25°C/60% RH**	**22241**	**371**	**90884**	**120263**
	14	25°C/60% RH	651	284	485	2949
Orlistat, 5%w/w HPC-SL, 1.25%w/w (Hydroxypropyl Cellulose-Super Low Viscosity) Docusate Sodium, 0.05%w/w (Docusate Sodium) Deionised Water, 93.7%w/w	0	N/A	232	174	318	537
	0	N/A	263	217	371	532
	14	**5°C**	**40976**	**265**	**130336**	**174898**
	14	**5°C**	**3110**	**216**	**10063**	**22230**
	14	**25°C/60% RH**	**22255**	**170**	**84494**	**118656**
	14	25°C/60% RH	506	192	302	2102
Orlistat, 5%w/w HPC-SL, 2%w/w	0	N/A	419	330	639	971
	0	N/A	438	331	671	1100

Table 3						
Formulation	**Storage Time (days)**	**Storage Condition**	**Mean (nm)**	**D50 (nm)**	**D90 (nm)**	**D95 (nm)**
(Hydroxypropyl Cellulose-Super Low Viscosity) Deionised Water, 93%w/w	**14**	**5°C**	**76195**	**64261**	**175942**	**221539**
	14	**5°C**	**5418**	**399**	**19307**	**26735**
	14	**25°C/60% RH**	**29931**	**19086**	**78580**	**96406**
	14	25°C/60% RH	1211	310	3568	7161
Orlistat, 5%w/w Lutrol F127, 1.5%w/w (Poloxamer 407) Deionised Water, 93.5%w/w	0	N/A	347	205	436	1330
	0	N/A	265	192	314	617
	14	**5°C**	**6161**	**167**	**24954**	**46361**
	14	5°C	219	192	271	344
Orlistat, 5%w/w HPC-SL, 2%w/w (Hydroxypropyl Cellulose-Super Low Viscosity) Deionised Water, 93%w/w	0	N/A	232	174	318	537
	0	N/A	263	217	371	532
	14	**5°C**	**40976**	**265**	**130336**	**174898**
	14	**5°C**	**3110**	**216**	**10063**	**22230**
	14	**25°C/60% RH**	**22255**	**170**	**84494**	**118656**
	14	25°C/60% RH	506	192	302	2102

20. Accordingly, a stable nanoparticulate orlistat composition could not be obtained under the various conditions tested.

C. Banavath

21. The technologies employed to obtain nanoparticulate active agent compositions, such as precipitation, microemulsion, high pressure homogenization, and milling, are all associated with disadvantages. *See* Banavath et al., "Nanosuspension: An Attempt To Enhance Bioavailability Of Poorly Soluble Drugs," *Int'l J. Pharm. Sci. and Res.*, *1*(9): 1-11 (2011) (Exhibit 3), at page 4, Table 2.

22. More specifically, precipitation may cause the growth of drug crystals and requires that the drug be soluble in at least one solvent. Microemulsion requires the use of a high amount of surfactant and stabilizer, which increases production cost. At times, microemulsion even involves the use of hazardous solvents in production. Homogenization requires that the

drug be pre-processed into a micronized state, and possible contamination may occur from metal ions from the wall of the homogenizer. Milling is a time-consuming process which is hard to scale up and which may have contamination from the milling media. Also prolonged milling may induce instability of the drug, resulting in the drug transforming into an amorphous state. Therefore, not all active agents can be successfully made into nanoparticulate active agent formulations in view of the technologies available to date.

D. Wu

23. Wu et al. ("Physical and chemical stability of drug nanoparticles," *Advanced Drug Delivery Reviews*, electronically published in February, 2011, Exhibit 4) report that it remains challenging to obtain nanoparticulate active agent compositions that are physically and chemically stable because the stability is affected by many factors. *See* lines 84-105 and 855-861.

24. More specifically, Wu et al. teach that obtaining a stable nanoparticulate active agent composition is hindered by the difficulty of selecting a suitable surface stabilizer for the active agent. Moreover, according to Wu et al. the main challenges in designing nanoparticulate drug formulations are: (i) the lack of a fundamental understanding of the interaction between the surface stabilizer and the active agent nanoparticles (*see* lines 268-273); (ii) the process of selecting a surface stabilizer having an appropriate anchoring tail to the particular active agent is burdensome (*see* lines 268-273); (iii) the lack of predictability due to the lack of any correlation between the physiochemical properties of the active agent and the success rate of obtaining a stable nanoparticulate active agent composition (*see* lines 399-402); and (iv) the lack of an efficient and high throughput screening technique to identify a suitable surface stabilizer (*see* lines 812-816).

CONCLUSION

25. The data described herein demonstrate unpredictability in the art, such that there is no *a priori* expectation that any given active agent could be made into a nanoparticulate active agent composition, even when a functional equivalent of the active agent has successfully been made into a nanoparticulate active agent composition. Therefore, it is impossible to predict whether a nanoparticulate active agent composition can be made based on the success in making nanoparticulate compositions of other active agents in the same class.

26. I declare that the statements made herein of my knowledge are true and all statements on information and belief are believed to be true; and further these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing therein.

Gary G. Liversidge

2/8/12

Date

Enclosures: Publication by Kimura et al. (Exhibit 1);
Publication by Jinno et al. (Exhibit 2);
Publication by Banavath et al (Exhibit 3);
Publication by Wu et al. (Exhibit 4);

EXHIBIT 1

PubMed "Arzneimittel-Forschung"[Jour] AND 35[volume] AND 1144 Search

Display Settings: Abstract

Arzneimittelforschung. **1985**;**35**(7A):**1144**-9.

Effect of cilostazol on platelet aggregation and experimental thrombosis.

Kimura Y, Tani T, Kanbe T, Watanabe K.

Abstract

A new antithrombotic drug, cilostazol (6-[4-(1-cyclohexyl-1 H-tetrazol-5-yl)butoxy]-3,4-dihydro-2(1H)-quinolinone, OPC-13013) was studied for its inhibitory effect on platelet aggregation in vitro in various experimental animals and man and in dogs ex vivo, for its effect to disperse platelet aggregates in vitro in rabbits and man and for its antithrombotic effect in vivo using its effect to prevent death due to the formation of pulmonary thrombi in mice. Cilostazol produced a potent inhibition of platelet aggregation both in vitro and ex vivo and a dispersion of platelet aggregates in vitro. The mode of action of cilostazol was different from that of acetylsalicylic acid (ASA) in that cilostazol inhibits not only secondary platelet aggregation but also primary platelet aggregation induced by aggregating agents such as adenosine diphosphate (ADP). The drug potently prevented death due to pulmonary thrombosis by platelet aggregates in mice in vivo. Unlike ASA which prevented only death due to collagen-induced platelet aggregation, cilostazol prevented both collagen- and ADP-induced platelet aggregation. These results suggest that cilostazol is a promising antithrombotic drug.

PMID:4074426[PubMed - indexed for MEDLINE]

Publication Types, MeSH Terms, Substances

LinkOut - more resources

EXHIBIT 2

Available online at www.sciencedirect.com
SCIENCE DIRECT
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Effect of particle size reduction on dissolution and oral absorption of a poorly water-soluble drug, cilostazol, in beagle dogs

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Abstract

The purpose of the present study was to investigate the effects of particle size on the dissolution and oral absorption of cilostazol. Three types of suspensions having different particle size distributions were prepared of the hammer-milled, the jet-milled cilostazol crystals and the NanoCrystal® spray-dried powder of cilostazol. In vitro dissolution rate of cilostazol was significantly increased by reducing the particle size. The dissolution curves of the cilostazol suspensions were in good agreement with the simulation based on the Noyes–Whitney equation. The bioavailability of cilostazol after oral administration to dogs was increased with reducing the particle size. While positive food effect on the absorption was observed for the suspensions made of the hammer-milled and the jet-milled crystals, no significant food effect was found for the suspension made of the NanoCrystal® cilostazol spray-dried powder. These results could be qualitatively predicted from the in vitro dissolution data using the bio-relevant media, FaSSIF and FeSSIF. In conclusion, the NanoCrystal® technology is found to be efficient to improve the oral bioavailability of cilostazol and to avoid the food effect on the absorption.
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Keywords: Dissolution; Bioavailability; Particle size reduction; Food effect; NanoCrystal; Cilostazol

1. Introduction

Cilostazol is a synthetic antiplatelet agent with vasodialating effect [1]. This drug is approved for a treatment of ischemic symptoms related to peripheral arterial occlusive diseases in Japan and several other countries as Pletaal® tablet, and for a treatment of intermittent claudication in U.S.A. and U.K. as Pletal® tablet [2–5]. Recent study proved that cilostazol is also effective for a prevention of recurrence of cerebral infarction [6]. The molecular weight and melting point of cilostazol are 369.47 and 159.4–160.3 °C, respectively. Cilostazol is a neutral molecule having an aqueous solubility of 3 μg/mL at 25 °C [7]. Octanol–water distribution coefficients ($\log P_{oct}$) of the drug ranged from 2.72 (pH 2.0) to 2.76 (pH 11.0) [7]. An apparent permeability of cilostazol through Caco-2 cell monolayer was found to be 1.92×10^{-5} cm/s [8]. Therefore, according to Biopharmaceutics Classification System (BCS) [9], cilostazol is categorized in Class II (poorly soluble and highly permeable). Fraction dose absorbed of cilostazol from a suspension in 5% ethanol in rats or dogs was found to be 88.0% at 10 mg/kg or 50.7% at 3 mg/kg, respectively, calculated from the recovery of unabsorbed drug in feces [10,11]. The area under the serum concentration–time curve (AUC) of cilostazol for the 50 mg tablet was found to be significantly less (−13%) than that for ethanolic solution in humans. A shorter half-life ($t_{1/2z}$) of cilostazol at the apparent terminal elimination phase after dosing the ethanolic solution (2.5±0.4 h) than that of the tablet (11.0±4.0 h) suggested that the absorption rate constant from the tablet was smaller than the elimination rate constant. These results suggest that the incomplete absorption of cilostazol from the tablet in humans was likely due to the poor dissolution [12].

It is well known that poorly water-soluble drugs often exhibit increased or accelerated absorption when they are

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administered with food [13]. This positive food effect would be attributed to the enhancement of the dissolution rate in the gastrointestinal (GI) tract caused by many factors such as delayed gastric emptying, increased bile secretion, larger volume of the gastric fluid, increased gastric pH (for acidic drugs) and/or increased splanchnic blood flow. In fact, a standard high fat breakfast increased both the rate (+91%) and extent (+24%) of cilostazol absorption after an oral administration of the 100 mg tablet [12], suggesting that the oral bioavailability of cilostazol could be enhanced due to the improvement of dissolution by food.

The dissolution rate of a solid drug can be expressed by the Noyes–Whitney equation [14], and it is also well known that the dissolution rate can be proportionally increased by increasing surface area as a consequence of comminution. Mechanical milling is a common technique to enhance dissolution of poorly water-soluble drugs [15]. Impact mills such as a hammer-mill or fluid energy mills such as a jet-mill are generally used for micronization of active ingredient in pharmaceutical industry [16]. In general, the former produces particles having mean diameters greater than 10 μm and the latter provides particles approximately ten times smaller than the hammer-milled particles. However, it is difficult to reduce particle size in sub-micron region using these dry-mills. NanoCrystal® is an enabling technology to produce sub-micron particles by wet-milling [17–19]. In this technology, materials are grinded with milling beads in water containing steric- and charge-stabilizers to prevent irreversible agglomeration of the resulted sub-micron particles. Anionic surfactants and hydrophilic polymers are used as the charge-stabilizers and the steric-stabilizers, respectively. Significant enhancement of oral bioavailability by this technology was reported for some BCS Class II compounds [17,19]. Elimination of positive food effect was also reported as an advantage of NanoCrystal® [19].

The active ingredient of the commercial formulations is sized with a hammer-mill, resulting in mean particle diameter greater than 10 μm. Oral bioavailability of cilostazol, therefore, is thought to be improved by extensive particle size reduction with a jet-mill or a media-mill (NanoCrystal®). The purpose of the present study was to investigate the effects of particle size on the dissolution rate as well as the rate and the extent of oral absorption of cilostazol. Food effect on the absorption was also investigated.

2. Materials and methods

2.1. Materials

Cilostazol and an internal standard OPC-13012 (6-[4-(1-cyclohexyl-1*H*-tetrazol-5-yl)propoxy]-3,4-dihydro-1-ethyl-2(1*H*)-quinolinone) were synthesized in Otsuka Pharmaceutical Co., Ltd. (Tokushima, Japan). Sodium taurocholate and egg lecithin (biochemistry grade) were obtained from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan) and Kanto Chemical (Tokyo), respectively. All other reagents were analytical grade commercial products.

2.2. Particle size reduction of cilostazol

The hammer-milled cilostazol crystal was prepared with Atomizer AIIW5G (Dalton, Tokyo) and the jet-milled cilostazol crystal was prepared with Super Sonic Jet Mill PJM-100SP (Nippon Pneumatic MFG Co., Ltd. Osaka, Japan). The NanoCrystal® cilostazol spray-dried powder, containing 16.5% of hydroxypropyl cellulose and 0.8% docusate sodium was prepared by spray drying of wet-milled cilostazol dispersion prepared with Dyno®-Mill (type KDL, Glen Mills, Inc., Clifton, NJ, USA). The NanoCrystal® cilostazol spray-dried powder exhibited excellent re-dispersibility in water, the simulated gastric and intestinal fluids in USP. X-ray diffraction analysis indicated that the crystal form of cilostazol was not changed by the milling procedures. No chemical degradation was found by the treatments.

Particle size distributions of the milled cilostazol crystals were determined with a laser diffraction particle size analyzer, SALD-3000J (Shimadzu, Kyoto, Japan), in 0.5% hydroxypropyl methylcellulose aqueous solution as a dispersing medium.

2.3. Preparation of cilostazol suspensions

The NanoCrystal® cilostazol suspension was prepared by dispersing the NanoCrystal® cilostazol spray-dried powder in water at 2.5 mg/mL. The hammer-milled and the jet-milled cilostazol suspensions were prepared with an aqueous solution containing hydroxypropyl cellulose and docusate sodium at the same contents of those in the NanoCrystal® suspension at the same concentrations.

2.4. Preparation of simulated intestinal fluids

The simulated intestinal fluids in the fasted state (FaSSIF) and the fed state (FeSSIF) [20] were utilized as dissolution media in order to predict in vivo dissolution and the food effect on cilostazol absorption. FaSSIF contains 3 mM sodium taurocholate and 0.75 mM lecithin, adjusted at pH 6.5. FeSSIF contains 15 mM sodium taurocholate and 3.75 mM lecithin, adjusted at pH 5.0.

2.5. Solubility measurement

Equilibrium solubility values of cilostazol at 37 °C were determined in water, FaSSIF and FeSSIF. Excess amount of the jet-milled cilostazol crystal was added in each medium in a screw-cap vial. Then, the vials were shaken continuously in a water bath maintained at 37 °C for 24 h. The equilibrated samples were immediately filtered through a 0.2 μm membrane filter, and the filtrate was diluted with appropriate volume of methanol. A 50-μL volume of the sample was analyzed by a reversed-phase HPLC method.

The solubility values of the hammer-milled cilostazol crystal and the NanoCrystal® cilostazol spray-dried powder were estimated from measured values of the jet-milled

crystal using the Ostwald–Freundlich equation [14,21,22] (Eq. (1)).

$$\ln\frac{C_s}{C_{s0}} = \frac{2v\gamma}{rRT} = \frac{2M\gamma}{\rho rRT} \quad (1)$$

where r, v, γ, R and T mean the radius of spherical drug particle, molar volume, interfacial energy, ideal gas constant and absolute temperature, respectively. ρ and M are the density and molecular weight, respectively. C_s is the solubility of the spherical particle, and C_{s0} is the solubility of a flat solid sheet.

2.6. In vitro dissolution profile measurement

In vitro dissolution profiles of the cilostazol suspensions were determined in 900 mL of water, FaSSIF and FeSSIF as dissolution media at 37 °C by USP Apparatus 2 using DT-610 dissolution tester (JASCO, Tokyo). Dissolved cilostazol was quantified from absorbance difference between the wavelengths of 257 nm and 325 nm. Based on the solubility values obtained in the previous section, 5 mg of cilostazol was applied as maximum amount to be solubilized in 900 mL of water. As a preliminary study showed that the dissolution profiles obtained at the speeds of 50 and 200 rpm were equivalent (data not shown), it was fixed at 50 rpm in this research.

2.7. Bioavailability studies in beagle dogs

Cilostazol was orally administered to four beagle dogs (body weight 8–10 kg) as the three types of oral suspensions, the hammer-milled, the jet-milled and the NanoCrystal® suspensions, at 100 mg/body in crossover design. A washout period of 1 week was kept between consecutive dosings. The dogs were fasted for 23 h before dosing and for 10 h post-dosing as a fasted condition. For the fed condition, the dogs were fasted for 22 h until 30 min prior to the dosing, and were then given 170 g of a solid food (CLEA Dog Diet CD-5M containing 24% protein and 9% fat, 635 kcal, CLEA JAPAN, Inc., Tokyo). The complete consumption of the food was ensured every time before dosing. Cilostazol dissolved in 5% DMSO was also intravenously administered to another group of four male beagle dogs (body weight 8–10 kg) at 10 mg/0.2 mL/kg. Dogs were allowed free access to water throughout the experiment. Blood samples (1.5 mL) were collected from a forearm vein with heparinized syringes at 0 (pre-dose), 0.5, 1, 2, 3, 4, 6, 8 and 10 h post-dose for the oral administration and 0 (pre-dose), 0.5, 1, 2, 5, 15, 30, 60 and 120 min post-dose for the intravenous administration. Serum samples were obtained by centrifugation of the blood samples and stored at −20 °C until use. Our investigations were performed after approval by our local ethical committee at Otsuka Pharmaceutical Co. Ltd. and Okayama University and in accordance with "Principles of Laboratory Animal Care" (NIH Publication # 85 - 23).

2.8. Analytical method

2.8.1. Cilostazol in the simulated intestinal fluids

Samples of solubility measurement were introduced onto an HPLC system, consisting of an HPLC pump (Model LC-10A, Shimadzu, Kyoto) and a UV detector (Model SPD-10A, Shimadzu) set at 254 nm. A C18 column (TSK gel ODS-80Ts, 4.6 mm i.d. × 150 mm, Tosoh, Tokyo) was used as an analytical column. A mobile phase containing 0.2% w/v sodium lauryl sulfate and 0.03% v/v phosphoric acid in acetonitrile–methanol–water mixture (3:3:4, v/v/v) was delivered at 1.0 mL/min. Coefficient of variation of standard curve ranged from 0.24% to 1.15% and the correlation coefficient was over 0.999.

2.8.2. Cilostazol in serum

Cilostazol in serum was determined by validated HPLC methods using OPC-13012 as an internal standard. A 200 μL volume of the internal standard solution (1 μg/mL in acetonitrile) was added to a 200 μL volume of the serum sample diluted with blank serum if it was needed. Then it was mixed well with a vortex mixer and stood for 20 min at the room temperature. A 200 μL volume of water was added to the mixture and mixed well with the vortex mixer. The mixture was centrifuged at 1800×*g* for 10 min, then the supernatant was filtered through a 0.45-μm membrane filter. The samples were applied to an HPLC system, consisting of an HPLC pump (Model Nanospace 3001, Shiseido, Tokyo) and a UV detector (Model Nanospace 3023, Shiseido) set at 254 nm. A 100 μL of the resulting filtrate was injected to the pre-column, TSK pre-column BSA–ODS (4.6 mm i.d. × 10 mm, Tosoh), adjusted at 40 °C and the pre-column was washed with the mobile phase for purification (1:10 diluted phosphate-buffered saline (−)) for 2 min at the flow rate of 500 μL/min, then it was back flushed to the analysis column, Capcell Pak C18 MG S-3 μm (3.0 mm i.d. × 75 mm, Shiseido) adjusted at 40 °C with the mobile phase for analysis (a mixture of acetonitrile–water (2:3 v/v) containing 0.3% v/v of THF) for 5 min at 300 μL/min. The chromatograms were obtained at the detection wavelength of 254 nm. Calibration curves (10–1000 ng/mL, 10 concentrations) and quality control (QC) samples (20, 200 and 1000 ng/mL) were freshly prepared for each analysis. The lower limit of quantification by this method was 10 ng/mL. The linear regression coefficients of the calibration curves ranged from 0.99 to 0.9999. The accuracy and the precision of the QC samples ranged from 92.0% to 118.0% from the nominal values, and 2.3% to 14.5% CV, respectively. Several samples were assayed by using the HPLC system, consisting of the HPLC pump (Model LC-10A, Shimadzu) and the UV detector (Model SPD-10A, Shimadzu) set at 254 nm. TSK precolumn BSA–ODS (4.6 mm i.d. × 35 mm, Tosoh) and YMC AM-302 S-5 μm (4.6 mm i.d. × 150 mm, YMC, Kyoto) were used as a pre-column and an analytical column, respectively. The linear regression coefficients of the calibration curves (10–1000 ng/mL) were higher than 0.9999. The accuracy and the precision of the QC samples ranged from 96.4% to 109.0% from the nominal values, and 0.7% to 5.8% CV, respectively.

2.9. Pharmacokinetic analysis

The highest serum concentration of cilostazol was employed as C_{max}, and the time to reach C_{max} was defined as t_{max}. AUC and the area under the first-moment curve, AUMC, were calculated from 0 to infinity using a linear trapezoidal rule. The serum concentration at time zero (C_0) for intravenous administration was estimated by extrapolation. The mean residence time, MRT, was calculated by AUMC/AUC. The absolute bioavailability (F) was calculated based on Eq. (2).

$$F = \frac{AUC_{oral}}{AUC_{i.v.}} \times \frac{Dose_{i.v.}}{Dose_{oral}} \quad (2)$$

where AUC_{oral} and $Dose_{oral}$ are AUC at infinite time and dose for oral administration of cilostazol. $AUC_{i.v.}$ and $Dose_{i.v.}$ mean AUC and dose for intravenous administration of cilostazol.

The in vivo absorption rate of cilostazol was estimated using a numerical deconvolution technique. The mean serum concentration data from the oral administration study were assigned as a response function, while the data from the intravenous administration study were used as a weight function.

2.10. Simulation of in vitro dissolution

In vitro dissolution of the cilostazol suspensions was simulated by the method reported by Hints and Johnson [23], where they expanded a mixing tank model for monodispersed solid drug [24] to polydispersed particles [23,25,26]. Assuming that spherical particles have size fractions $i=1$ to n, that the number of particles dose not change with time and that the diffusion layer thickness at time t is equal to the particle radius at time t, the rate of change in the mass of remaining solid of the particle size fraction i is given by Eq. (3):

$$\frac{dX_{s_i}}{dt} = -\frac{3DX_{0_i}^{2/3}X_{s_i}^{1/3}}{\rho r_{0_i}^2}\left(C_s - \frac{X_{dT}}{V}\right) \quad (3)$$

where X_0 is the initial mass of the drug, X_{si} is the mass of the remaining solid drug at time t for size fraction i. X_{dT} is the total mass of the dissolved drug at time t for the size fractions $i=1$ to n. D and ρ mean the drug diffusivity in aqueous phase and the density of the drug, respectively. r_{0i} is the initial particle radius of particle size for fraction i. C_s and V are the solubility of the drug and fluid volume, respectively. The rate of change in the mass of dissolved drug is given by Eq. (4).

$$\frac{dX_{d_i}}{dt} = \frac{3DX_{0_i}^{2/3}X_{s_i}^{1/3}}{\rho r_{0_i}^2}\left(C_s - \frac{X_{dT}}{V}\right) \quad (4)$$

where X_{d_i} represents the mass of dissolved drug for size i at any time.

The total masses of the remaining solid drug, X_{sT}, and dissolved drug, X_{dT}, at time t for the size fractions $i=1$ to n were described by Eqs. (5) and (6), respectively.

$$X_{sT} = \sum_{i=1}^{n} X_{s_i} \quad (5)$$

$$X_{dT} = \sum_{i=1}^{n} X_{d_i} \quad (6)$$

X_{sT} and X_{dT} were simulated from Eqs. (3)–(6) by the 4th Runge–Kutta numerical integration method using a computer software, Mathematica 5.0 (Wolfram Research Inc., Champaign, IL, USA). The true density of cilostazol was determined with a gas pycnometer, Accupyc 1330 (Micromeritics, Norcross, GA, USA). The diffusivity of cilostazol in water was estimated by a method reported by Hyduk and Laudie [27] using a molar volume estimated by Schroeder's method [28].

2.11. Statistical analysis

Statistical analysis of the effects of particle size and food were performed by Welch's test using PSAG-CP® software (ASmedica, Osaka). P-value less than 0.05 was considered significant.

3. Results and discussion

3.1. Size distribution of milled cilostazol

Particle sizes of the three different types of milled cilostazol were measured and the size distributions are shown in Fig. 1. The median particle diameters of cilostazol in the hammer-milled, the jet-milled and the NanoCrystal® suspensions were found to be 13, 2.4 and 0.22 μm, respectively.

3.2. Solubility

Equilibrium solubility values of the jet-milled cilostazol crystal at 37 °C were determined to be 6.26±0.06, 6.35±0.12 and 12.7±0.2 μg/mL in water, FaSSIF and FeSSIF, respectively. The solubility in FeSSIF superior to that in FaSSIF or water could be attributed not to pH but to micellar solubilization due to higher concentration of sodium taurocholate and lecithin, since cilostazol is a neutral lipophilic compound. The solubility values of the hammer-milled cilostazol crystal and the NanoCrystal® cilostazol spray-dried powder were estimated to be practically identical (0.4% less) and 6% greater than those of the jet-milled cilostazol crystal, respectively. The parameters

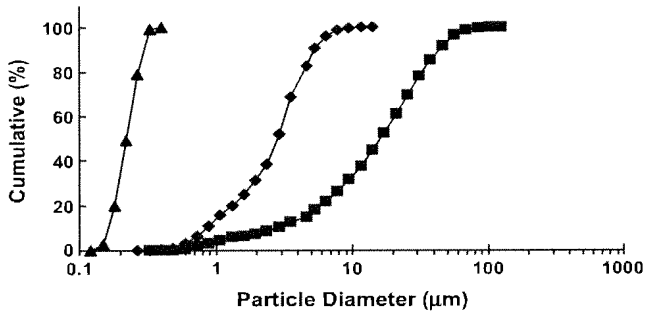

Fig. 1. Particle size distribution of cilostazol suspensions. Keys: ▲, NanoCrystal® spray-dried powder; ◆, jet-milled crystal; ■, hammer-milled crystal.

Table 1
Parameters for dissolution simulation

Parameter	Value
X_0: initial dose	5 mg
C_s: solubility[a]	6.26 μg/mL (in water)
	6.35 μg/mL (in FaSSIF)
	12.7 μg/mL (in FeSSIF)
D_{eff}: diffusivity	4.04×10^{-4} cm²/min
ρ: density	1.26 g/cm³
V: fluid volume	900 mL
Integration step	0.1 min[b]
	0.01 min[c]

[a] For the hammer-milled and the NanoCrystal® suspension, these values were multiplied by 0.996 and 1.06, respectively, according to the Ostwald–Freundlich equation.
[b] For the hammer-milled and the jet-milled suspension.
[c] For the NanoCrystal® suspension.

used for this calculation are as follows: M=369.47 g/mol, γ=30 mN/m, ρ=1.26 g/cm³, R=8.314 × 10⁷ g cm²/s²/K/mol, T=310 K [22].

3.3. In vitro dissolution rate

In vitro dissolution of cilostazol from the suspension consisting of the hammer-milled, the jet-milled crystals or the NanoCrystal® cilostazol spray-dried powder was investigated in water and the bio-relevant media, FeSSIF and FaSSIF. Solubility of cilostazol in these media is not enough to solubilize clinical doses (50 mg or 100 mg) in 900 mL as shown in the solubility study. Therefore, 5 mg of cilostazol was applied as maximum amount to be solubilized in 900 mL of water, FeSSIF or FaSSIF. Furthermore, the simulation method was also employed to assess the dissolution of cilostazol from the suspensions. The parameters used for the simulation are listed in Table 1. Results shown in Fig. 1 were used as data of particle size distribution. Solubility values obtained in the previous section were also used for the simulation.

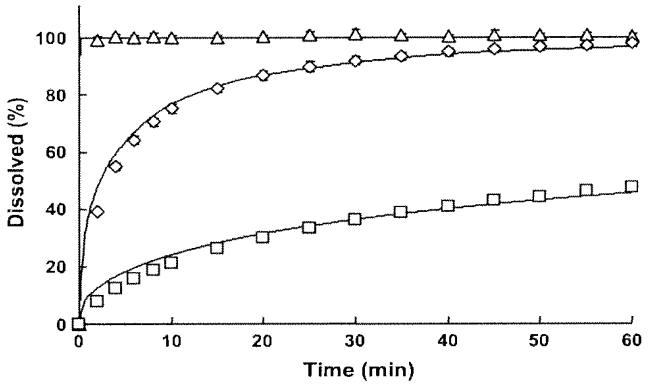

Fig. 2. In vitro dissolution profiles of cilostazol from the suspensions in water at 37 °C. Dissolution study was performed at 50 rpm following USP Apparatus 2. About 5 mg of cilostazol was applied in 900 mL water. Results are expressed as the mean with the bar showing S.D. values of six experiments and simulated curves (solid lines). Keys: △, NanoCrystal® spray-dried powder; ◇, jet-milled crystal; □, hammer-milled crystal.

Table 2
Simulated 50% dissolution time ($T_{50\%}$, min) of cilostazol suspensions at 37 °C

Formulation	Dissolution medium		
	Water	FaSSIF	FeSSIF
NanoCrystal®	0.016	0.016	0.0068
Jet-milled	2.3	2.3	0.97
Hammer-milled	82	80	32

Calculated from simulation results.

3.3.1. Dissolution in water

The dissolution profiles of the cilostazol suspensions in water are shown in Fig. 2. The dissolution rate of cilostazol from the suspensions was clearly affected by the particle size of the active ingredient. From the hammer-milled suspension, only 45% of applied cilostazol was dissolved in 60 min. In the case of the jet-milled suspension, more than 80% of cilostazol was dissolved within 15 min and the dissolved fraction reached 95% in 60 min. In contrast, the dissolution of cilostazol from the NanoCrystal® suspension was completed immediately. As sub-micron particles such as NanoCrystal® cilostazol can easily pass through a line filter, it is possible to overestimate the dissolution rate. A high-speed centrifugation method [29], therefore, was attempted to separate the solid particles (data not shown), but it was not appropriate because of considerably long time to separate them and the difficulty in controlling temperature during the centrifugation. Although the possible overestimation cannot be excluded, the simulated line agreed with the actual data very well, indicating the extremely rapid dissolution of the NanoCrystal® cilostazol suspension. Visual observation of immediate disappearance of turbidity in the dissolution vessel also suggested that the solid particles passed through the filter could be ignored.

The excellent agreement between the observed data and the simulated dissolution curves suggested that the dissolution of cilostazol from the suspensions followed the Noyes–Whitney equation and that the model with the assumptions and the parameters used were appropriate. The results shown in Fig. 2

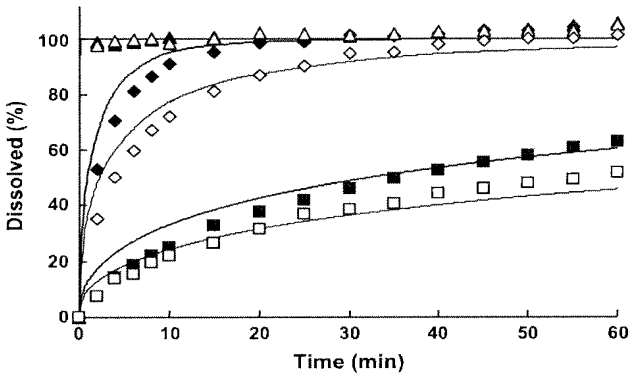

Fig. 3. In vitro dissolution profiles of cilostazol from the suspensions in the bio-relevant media at 37 °C. Dissolution study was performed at 50 rpm following USP Apparatus 2. About 5 mg of cilostazol was applied in 900 mL water. Results are expressed as the mean of two experiments and simulated curves (fine lines for FaSSIF and bold lines for FeSSIF). Keys: In FaSSIF: △, NanoCrystal® spray-dried powder; ◇, jet-milled crystal; □, hammer-milled crystal; In FeSSIF: ▲, NanoCrystal® spray-dried powder; ◆, jet-milled crystal; ■, hammer-milled crystal.

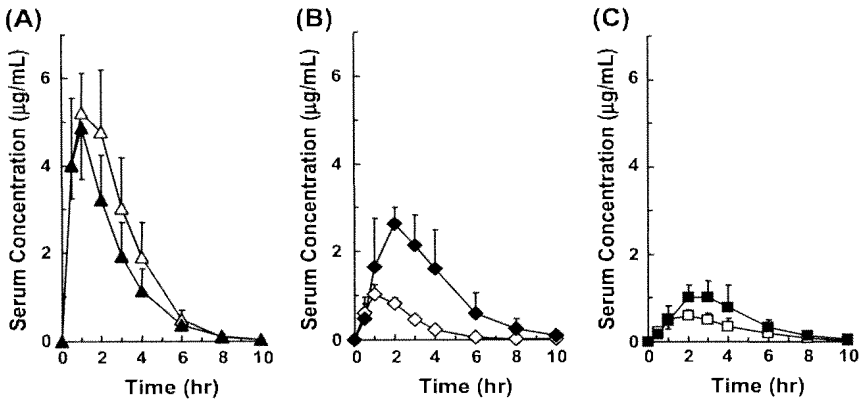

Fig. 4. Serum concentration–time profiles of cilostazol after oral administration of the suspensions at a dose of 100 mg/body in beagle dogs. Results are expressed as the mean with the bar showing S.D. values of four experiments. Keys: (A) △, NanoCrystal® suspension (fasted); ▲, NanoCrystal® suspension (fed); (B) ◇, jet-milled suspension (fasted); ◆, jet-milled suspension (fed); (C) □, hammer-milled suspension (fasted); ■, hammer-milled suspension (fed).

were much better than those obtained when cilostazol was treated as monodispersed particles with a mean particle size (data not shown).

Assuming smooth spherical particles, specific surface area is inversely proportional to particle size. Accordingly, the specific surface area of the jet-milled cilostazol crystal and the cilostazol crystal in the NanoCrystal® spray-dried powder were calculated as 3.2- and 28-fold greater than that of the hammer-milled crystal, treated as spherical polydispersed particles having size distribution shown in Fig. 1. Based on the simulated curves, 50% dissolution times ($T_{50\%}$) of the hammer-milled, the jet-milled and the NanoCrystal® suspensions were calculated to be 82 min, 2.3 min and 0.016 min, respectively (Table 2), indicating that the dissolution rates of the jet-milled and the NanoCrystal® suspensions would be 36-fold and 5100-fold greater than that of the hammer-milled suspension, respectively.

3.3.2. Dissolution in bio-relevant media

In order to predict the food effect on the absorption of cilostazol from the suspensions, the dissolution was investigated in the bio-relevant media, FaSSIF and FeSSIF (Fig. 3), because food effects on the extent of absorption of BCS Class II drugs such as danazol and atovaquone were well correlated with the FeSSIF/FaSSIF ratio of the in vitro dissolved amount [20,30–32]. The data obtained from two vessels for each suspension were superimposed, and the simulated lines agreed fairly well with the observed data. The dissolution rates of cilostazol in FaSSIF were similar to, and those in FeSSIF were faster than those obtained in water for all the suspensions. Simulation study showed that the $T_{50\%}$ values for the hammer-milled, the jet-milled and the NanoCrystal® cilostazol suspensions were 80, 2.3 and 0.016 min in FaSSIF, and 32, 0.97 and 0.0068 min in FeSSIF, respectively (Table 2). Although the FeSSIF/FaSSIF ratio of $T_{50\%}$ for each suspension was around 0.4 regardless of the difference in particle size, the difference in the absolute value of $T_{50\%}$ between FeSSIF and FaSSIF became much smaller as the particle size reduced. Especially, for the NanoCrystal® suspension, it can practically be neglected. These results suggest that the food effect can be avoided by the enhancement of dissolution rate due to the reduction of particle size.

3.4. Bioavailability study in dogs

The serum concentration–time profiles and the pharmacokinetic parameters of cilostazol resulted from the oral admin-

Table 3
Pharmacokinetic parameters

Food condition	Parameter	Intravenous administration (10 mg/kg)	NanoCrystal® (100 mg/body)	Jet-milled (100 mg/body)	Hammer-milled (100 mg/body)
Fasted	C_{max} (ng/mL)	–	5371±1173***	1029±218	582±154
	AUC (ng h/mL)	17584±7556	17832±4994**	2875±587	2722±803
	t_{max} (h)	–	1.3±0.5	1.0±0.0	1.8±0.5
	MRT (h)	1.3±0.4	2.4±0.2**	2.3±0.2**	3.6±0.9
	F	1	0.86±0.29	0.15±0.04	0.14±0.05
Fed	C_{max} (ng/mL)	–	4872±1112***	2901±314**	1152±221
	AUC (ng h/mL)	–	13589±3895**	10669±3417*	4694±1612
	t_{max} (h)	–	1.0±0.0*	1.0±0.0	2.5±1.3
	MRT (h)	–	2.3±0.3*	3.4±0.8	3.7±0.6
	F	–	0.67±0.22	0.53±0.21	0.23±0.09

Results are expressed as the mean±S.D. of four experiments. ***$P<0.001$; **$P<0.01$; *$P<0.05$, compared to the corresponding parameters of the hammer-milled suspension.

CL_{total}, k_{el} and Vd_{ss} for the intravenous administration were calculated to be 5.4±1.8 L/h, 0.79±0.20 h^{-1} and 6.5±0.7 L, respectively.

Table 4
Fed/fasted ratio on pharmacokinetic parameters

Parameter	NanoCrystal® (100 mg/body)	Jet-milled (100 mg/body)	Hammer-milled (100 mg/body)
C_{max}	0.91±0.13	2.9±0.5***	2.0±0.3**
AUC	0.76±0.04	3.7±0.7**	1.8±0.6
t_{max}	0.88±0.25	1.8±0.5*	1.0±0.0
MRT	0.95±0.13	1.5±0.4*	1.1±0.4
F	0.78±0.04	3.6±0.7	1.8±0.6

Results are expressed as the mean±S.D. of four experiments. ***$P<0.001$; **$P<0.01$; *$P<0.05$, statistically significant difference between the fed state parameters and the fasted state parameters.

istrations of the suspensions in beagle dogs are presented in Fig. 4 and Table 3, respectively. To estimate the food effect, the fed/fasted ratios on the pharmacokinetic parameters were also listed in Table 4.

In the fasted condition, compared with the hammer-milled cilostazol suspension, the absorption of cilostazol was not improved so much by dosing the jet-milled suspension as shown in Fig. 4 and Table 3. On the other hand, the NanoCrystal® suspension significantly increased C_{max} and AUC of cilostazol 9.2 ($P<0.001$) and 6.7 times ($P<0.05$), respectively, resulting in 86% of the absolute bioavailability. These results indicated that the extensive reduction of particle size could lead to the improvement of bioavailability of cilostazol. Although it was expected that the jet-milled suspension also had improved the bioavailability of cilostazol based on the results of the in vitro dissolution study (Figs. 2 and 3), the absorption was not changed so much, which might be attributed to the difference in dissolution behavior between in vitro and in vivo.

In the fed condition, the C_{max} values of the hammer-milled and the jet-milled suspension were increased 2.0-fold ($P<0.01$) and 2.9-fold ($P<0.001$), respectively, and the AUC values were increased 1.8-fold (not statistically significant) and 3.7-fold ($P<0.01$), respectively (Table 3). These results clearly indicate the positive food effects on the rate and extent of cilostazol absorption for the hammer-milled and the jet-milled suspension. On the other hand, in the case of the NanoCrystal® suspension, the fed/fasted ratios of C_{max} and the AUC values were 0.91-fold (not statistically significant) and 0.76-fold (not statistically significant), respectively, suggesting slightly negative food effects for the NanoCrystal® suspension.

These results suggest that the in vivo dissolution of cilostazol from the hammer-milled and the jet-milled suspension was improved by the food intake, which could be supported by the absorption rate–time profiles of cilostazol after oral dosing (Fig. 5). As cilostazol is classified into Class II of BCS, the absorption could be rate-limited by the dissolution process. Therefore, the profiles shown in Fig. 5 could reflect the in vivo dissolution behavior as well and clearly indicate that the food intake enhanced and prolonged the dissolution and absorption of cilostazol in the cases of the hammer-milled and the jet-milled suspensions. The prolongation of the dissolution, which is also suggested by the values of MRT (Table 3), might be ascribed to the improvement of dissolution by the food and/or bile and to the delay of gastric emptying by the food [33]. On the other hand, the dissolution and absorption of cilostazol from the NanoCrystal® suspension were not enhanced and not prolonged by the foods (Table 3 and Fig. 5) because the dissolution might be maximally improved by the size reduction and the drug might partially adsorb to the ingested food. Fig. 5 also shows that initial dissolution/absorption rates in 1 h after administration for the hammer-milled, the jet-milled and the NanoCrystal® cilostazol suspensions were 4.9%, 10.1% and 51.7% in the fasted condition, 4.6%, 14.2% and 50.5% in the fed condition, respectively. These results clearly indicated that the smaller particle resulted in the faster dissolution/absorption. However, it was also shown that food did not enhance the apparent initial absorption rate, which might be explained by the delay of gastric emptying by the food intake [33]. As gastric emptying is another possible rate-limiting step for the absorption, the retardation of gastric emptying could cancel out the potency of the dissolution enhancement.

The particle size reduction certainly enhanced the in vitro dissolution rate and the in vivo dissolution/absorption of cilostazol, but the relationship in the enhancement of dissolution between in vitro and in vivo was not necessarily

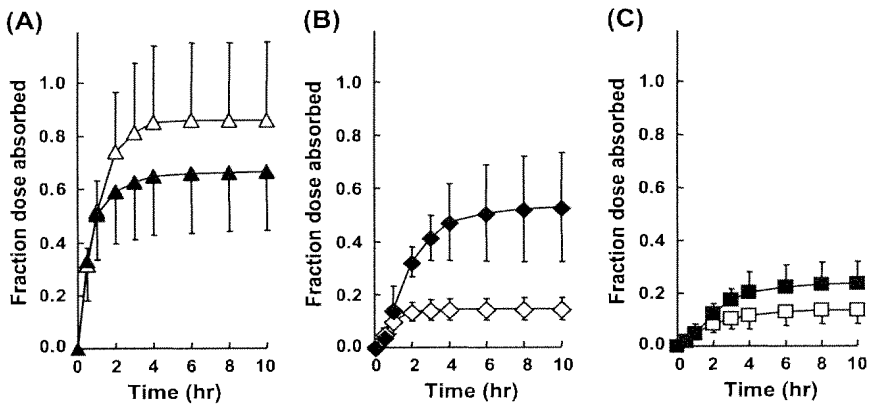

Fig. 5. Absorption rate–time profiles of cilostazol after oral administration of suspensions at a dose of 100 mg/body in beagle dogs. Results are expressed as the mean with the bar showing S.D. values of four experiments. Keys: (A) △, NanoCrystal® suspension (fasted); ▲, NanoCrystal® suspension (fed); (B) ◇, jet-milled suspension (fasted); ◆, jet-milled suspension (fed); (C) □, hammer-milled suspension (fasted); ■, hammer-milled suspension (fed).

quantitative. The initial dissolution/absorption rates described above, based on the results shown in Fig. 5, are also obviously larger than the cumulative dissolved amounts for 1 h in the in vitro dissolution study (Fig. 3). It would be difficult to find out the quantitative relationship between in vivo dissolution and in vitro dissolution by a simple dissolution study as employed in the present study, because such a dissolution study is not able to reflect the maintenance and enhancement of the dissolution, which could be ascribed to the sink condition that might be kept to some extent by the sequential absorption right after dissolution in vivo.

In the case of the hammer-milled suspension, which might correspond to the commercial tablet in human study, the bioavailability of cilostazol was enhanced around twice by the food intake, which is in good agreement with the human data where C_{max} and AUC of cilostazol after dosing the commercial 100 mg tablet were 1.7- to 1.9-fold and 1.2- to 1.3-fold greater, respectively, in the fed condition than those in the fasted condition [12]. This food effect would be related with the FeSSIF/FaSSIF ratio in the dissolution rate and/or solubility. This might also be the case with the jet-milled suspension. Although the food effect was a little more significant for the jet-milled cilostazol than that for the hammer-milled one, the enhancement ratios in C_{max} and AUC observed for these suspensions were still comparable to the FeSSIF/FaSSIF ratio in the dissolution rate and/or solubility. On the other hand, the food intake did not increase the bioavailability of the NanoCrystal® cilostazol suspension. Taken all together, the food effect could be independent of the solubility, because the solubility and the FeSSIF/FaSSIF ratio in the solubility were almost same for all the three suspensions. The improvement of dissolution rate should be responsible for the improved bioavailability of cilostazol in the cases of the jet-milled and the hammer-milled suspensions. The reason why no food effect was observed for the NanoCrystal® cilostazol suspension might be that the dissolution rate of NanoCrystal® cilostazol is fast enough even under the fasted condition, where the absorption might be permeability-limited. Therefore, the further increase in the dissolution rate would not contribute to the improvement of the absorption.

4. Conclusion

In the present study, it was demonstrated that the in vitro dissolution rate of cilostazol from aqueous suspension was improved by milling, which was described by the Noyes–Whitney equation. The bioavailability of cilostazol was increased by the reduction of particle size, but remarkable enhancements with minimum food effect were observed for the NanoCrystal® cilostazol suspension, while moderate enhancement of bioavailability and significant food effect were seen in the absorption for the jet-milled suspension, compared to the suspension made of the conventional hammer-milled crystal. These findings clearly indicate that the bioavailability of cilostazol can be maximized and the food effect can be avoided by the NanoCrystal® technology for a robust formulation.

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EXHIBIT 3

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NANOSUSPENSION: AN ATTEMPT TO ENHANCE BIOAVAILABILITY OF POORLY SOLUBLE DRUGS

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ABSTRACT

Most of the new chemical entities coming out from High-throughput screening in drug discovery process are failing due to their poor solubility in the water. Poorly water-soluble drugs show many problems in formulating them in conventional dosage forms. One of the critical problems associated with poorly soluble drugs is too low bioavailability. The problem is even more complex for drugs belonging to BCS CLASS II category, as they are poorly soluble in both aqueous and organic media, and for those drugs having a log P value of 2. There are number of formulation approaches to resolve the problems of low solubility and low bioavailability. These techniques for solubility enhancement have some limitations and hence have limited utility in solubility enhancement. Nanotechnology can be used to resolve the problems associated with these conventional approaches for solubility and bioavailability enhancement. Nanotechnology is defined as the science and engineering carried out in the nanoscale that is 10^{-9} meters. The present article describes the details about nanosuspensions. Nanosuspensions consist of the pure poorly water-soluble drug without any matrix material suspended in dispersion. The review article includes the methods of preparation with their advantages and disadvantages, characterization and evaluation parameters and pharmaceutical applications. A nanosuspension not only solves the problems of poor solubility and bioavailability but also alters the pharmacokinetics of drug and thus improves drug safety and efficacy.

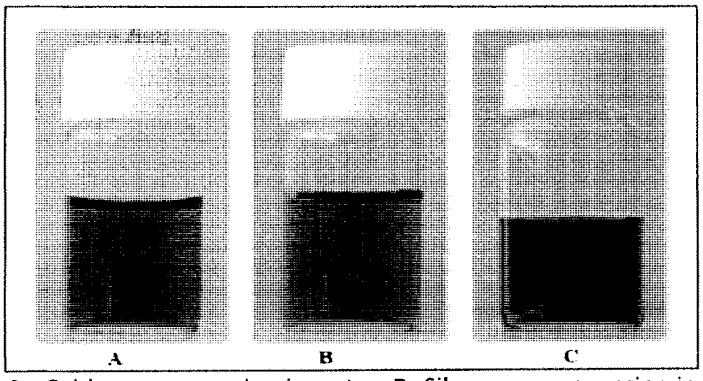
INTRODUCTION: Most of the new chemical entities (about 40%) coming out from High-throughput screening in drug discovery process are failing due to their poor solubility in the water [1]. As per a recent report [2], 46% of the total New Drug Applications (NDA) filed between 1995 and 2002 were BCS class IV, while only 9% were BCS class I drugs, revealing that a majority of the approved new drugs were water insoluble Because of their poor solubility it will become more complicated to incorporate them into the conventional dosage forms and thus decreasing the bioavailability of the drugs [3].

The problem is even more complex for drugs such as Glibenclamide (belonging to BCS CLASS II) as classified by BCS System [4] as they are poorly soluble in both aqueous and organic media, and for those drugs having a log P value of 2. For class II drugs, the rate limiting factor in their intestinal absorption is dissolution/solubility and thus the performance of these drugs is dissolution rate-limited and is affected by the fed/fasted state of the patient. Dissolution rates of sparingly soluble drugs are greatly affected by the shape as well as the particle size of the drug. Therefore decrease in particle size results in an increase in dissolution rate [5]. There are number of formulation approaches that can be used to resolve the problems associated with the low solubility and low bioavailability of these class II drugs. Some of the approaches to increase solubility include micronization [6], solubilisation using co-solvents, use of permeation enhancers, oily solutions, surfactant dispersions [6], salt formation [7] and precipitation techniques [8-9].

Most of these techniques for solubility enhancement have advantages as well as some limitations and hence have limited utility in solubility enhancement. Other techniques used for solubility enhancement like microspheres, emulsions, microemulsions [10], liposomes [11], super critical processing, solid-dispersions [12] and inclusion complexes using Cyclodextrins [13] show reasonable success but they lack in universal applicability to all drugs. These techniques are not applicable to the drugs, which are not soluble in both aqueous and organic Media.

However, there still remains an unmet need to equip the pharmaceutical industry with particle engineering technologies capable of formulating the poorly soluble drugs to improve their efficacy and to optimize therapy with respect to pharmacoeconomics. One such novel technology is nanosuspension technology. Nanosuspensions are sub-micron colloidal dispersions of nanosized drug particles stabilized by surfactants [14]. Nanosuspensions consist of the poorly water-soluble drug without any matrix material suspended in dispersion [15]. These can be used to enhance the solubility of drugs that are poorly soluble in aqueous as well as lipid media. As a result of increased solubility, the rate of flooding of the active compound increases and the maximum plasma level is reached faster.

This is one of the unique advantages that it has over other approaches for enhancing solubility. This approach is useful for molecules with poor solubility, poor permeability or both, which poses a significant challenge for the formulators. The reduced particle size renders the possibility of intravenous administration of poorly soluble drugs without any blockade of the blood capillaries. The suspensions can also be lyophilised and into a solid matrix. Apart from these advantages it is also having the advantages of liquid formulations over others. In the present review we are mainly focussing on the different methods of preparation, critical parameters and evaluation of the nanosuspension. Fig. 1 shows some of the nanosuspensions [16].



A- Gold nanosuspension in water, **B** -Silver nanosuspension in water, **C** -VOPc (vanadyl phthalocyanine) nanosuspension in water

FIGURE 1: FEW TYPES OF NANOSUSPENSIONS.

Nanosuspensions differ from nanoparticles [17] which are polymeric colloidal carriers of drugs (Nanospheres and nanocapsules), and from solid-lipid nanoparticles [18] (SLN), which are lipidic carriers of drug. The potential benefits of nanoparticles over conventional technologies are described in **Table 1** [19].

TABLE 1: POTENTIAL BENEFITS OF NANOSUSPENSION TECHNOLOGY

ROUTE OF ADMINISTRATION	POTENTIAL BENEFITS
Oral	• Rapid dissolution and • High bioavailability • Reduced fed/fasted ratio
Intravenous (I.V)	• Tissue targeting • Rapid dissolution • Longer duration of retention in systemic circulation
Ocular	• Higher bioavailability • Less irritation • More consistent dosing
Inhalation	• Higher bioavailability • More consistent dosing
Subcutaneous/ intramuscular	• Higher bioavailability • Rapid onset • Reduced tissue irritation

Preparation of Nanosuspensions: Preparation of nanosuspensions were reported to be a more cost effective and technically more simpler alternative than liposomes and other conventional colloidal drug carriers, particularly for poorly soluble drugs and yield a physically more stable product. The simplest method of preparation of nanosuspensions is micronization by colloid or jet milling [20], which improves the dissolution rate but is not having any effect on saturation solubility. Nanosuspension engineering processes currently used are preparation by precipitation, high pressure homogenization, emulsion and milling techniques. These techniques and the obtained compounds are summarized in **Table 2** and are briefly described in the following sections. Mainly there are two methods for preparation of nanosuspensions. The conventional methods of precipitation are called 'Bottom Up technology'. The 'Top Down Technologies' are the disintegration methods and are preferred over the precipitation methods. These include Media Milling (Nanocrystals), High Pressure Homogenization in water (Dissocubes), High Pressure Homogenization in nonaqueous media (Nanopure) and combination of Precipitation and High-Pressure Homogenization (Nanoedege). Few other techniques used for preparing nanosuspensions are emulsion as templates, microemulsion as templates etc.

- **Precipitation:** The most common method of precipitation used is anti solvent addition method in which the drug is dissolved in an organic solvent and this solution is mixed with a miscible antisolvent. Mixing processes vary considerably. Precipitation has also been coupled with high shear processing. The NANOEDGE process (is a registered trademark of Baxter International Inc. and its subsidiaries) relies on the precipitation of friable materials for subsequent fragmentation under conditions of high shear and/or thermal energy [32].

TABLE 2: SUMMARY OF THE NANOSUSPENSION FORMATION TECHNOLOGIES

Technology	Advantage	Disadvantage	Drug
Precipitation	Simple process. Ease of scale up. Economical production.	Growing of crystals needs to be limit by surfactant addition. Drug must be soluble at least in one solvent.	Carbamazepine [8] Cyclosporine [21] Griseofulvin [22]
Emulsion/Microemulsion template	High drug solubilization. Long shelf life. Ease of manufacture.	Use of high amount of surfactant and stabilizers. Use of hazardous solvent in production.	Breviscapine [23] Griseofulvin [24]
High pressure Homogenization	Applicable to most of the drugs Very dilute as well as highly concentrate nanosuspension can be prepared. Aseptic production possible.	High number of homogenization cycles. Drug should be in micronized state. Possible contamination could occur from metal ions coming off from the walls.	Albendazole [25] Aphidicolin [26] Azithromycin [27] Fenofibrate [28]
Milling methods • Media milling	Applicable to the drugs that are poorly soluble in both aqueous and organic media. Little batch to batch variation. High flexibility in handling large quantities of drugs.	Time consuming. Difficult to scale up. Prolonged milling may induce the formation of amorphous & instability.	Cilostazol [29] Danazol [3] Naproxen [3]
• Dry Co-grinding	Easy process and no organic solvent required. Require short grinding time.	Generation of residue of milling media.	Clarithromycin [30] Glibenclamide [31]

This is accomplished by a combination of rapid precipitation and high-pressure homogenization. Rapid addition of a drug solution to an antisolvent leads to sudden super saturation of the mixed solution, and generation of fine crystalline or amorphous solids. Precipitation of an amorphous material may be favored at high super saturation when the solubility of the amorphous state is exceeded. The success of drug nanosuspensions prepared by precipitation techniques has been reported in some journals [32-33].

- **Lipid Emulsion/Microemulsion Template:** Lipid emulsions as templates are applicable for drugs that are soluble in either volatile organic solvents or partially water miscible solvents. In this method the drug will be dissolved in the suitable organic solvent and then emulsified in aqueous phase using suitable surfactants. Then the organic solvent will be slowly evaporated under reduced pressure to form drug particles precipitating in the aqueous phase forming the aqueous suspension of the drug in the required particle size. Then the suspension formed can be diluted suitably to get nanosuspensions [34]. Moreover, microemulsions as templates can produce nanosuspensions. Microemulsions are thermodynamically stable and isotropically clear dispersions of two immiscible liquids such as oil and

water stabilized by an interfacial film of surfactant and co-surfactant. The drug can be either loaded into the internal phase or the pre-formed microemulsion can be saturated with the drug by intimate mixing. Suitable dilution of the microemulsion yields the drug nanosuspension [34]. An example of this technique is the griseofulvin nanosuspension which is prepared by the microemulsion [34]. The advantages of lipid emulsions as templates for nanosuspension formation are that they easy to produce by controlling the emulsion droplet and easy for scale-up. However, the use of organic solvents affects the environment and large amounts of surfactant or stabilizer are required.

- **High Pressure Homogenization:** It is the most widely used method for the preparation of the nanosuspensions of many poorly water soluble drugs [35-37]. Different methods developed based on this principle for preparation of nanosuspensions are *Dissocubes, Nanopure, Nanoedge, Nanojet technology*. In the high pressure homogenization method, the suspension of a drug and surfactant is forced under pressure through a nanosized aperture valve of a high pressure homogenizer.

The principle of this method is based on cavitation in the aqueous phase. The particles cavitations forces are sufficiently high to convert the drug microparticles into nanoparticles. The concern with this method is the need for small sample particles before loading and the fact that many cycles of homogenization are required [38-39]. Figure 2 gives the schematic representation of the high-pressure homogenization process

  - DissoCubes technology is an example of this technology developed by R.H. Müller using a piston-gap-type high pressure homogenizer, which was recently released as a patent owned by SkyePharm plc [34]. Scholer *et al.* prepared atovaquone nanosuspensions using this technique.
  - Nanopure is suspensions homogenized in water-free media or water mixtures.
  - Nanoedge is combination of precipitation and homogenization techniques resulting in smaller particle size and better stability in a shorter time.
  - *Nanojet technology*, also called as opposite stream, uses a chamber where a stream of suspension is divided into two or more parts, which colloid with each other at high pressure.

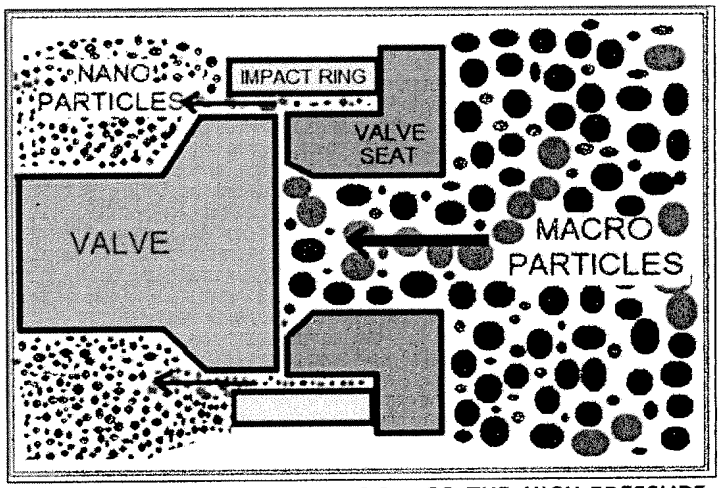


FIGURE 2: SCHEMATIC CARTOON OF THE HIGH-PRESSURE HOMOGENIZATION PROCESS

- **Milling Techniques:**
  - **Media milling:** Media milling is a further technique used to prepare nanosuspensions [24, 40]. This patent-protected technology was developed by Liversidge et al. [41]. Formerly, the technology was owned by the company NanoSystems but recently it has been acquired by Elan Drug Delivery. In this technique, the nanosuspensions are produced using high-shear media mills or pearl mills. The media mill consists of a milling chamber, a milling shaft and a recirculation chamber. The drug nanoparticles are obtained by subjecting the drug to media milling. High energy and shear

forces generated as a result of impaction of the milling media with the drug provide the necessary energy input to disintegrate the microparticulate drug into nanosized particles. The milling medium is usually composed of glass, zirconium oxide or highly cross-linked polystyrene resin. In batch mode, the time required to obtain dispersions with unimodal distribution profiles and mean diameters <200nm is 30–60 min. In the media milling process, the milling chamber is charged with the milling media, water or suitable buffer, drug and stabilizer. Then milling media or pearls are rotated at a very high shear rate.

- **Dry Co-Grinding:** Recently, nanosuspensions can be obtained by dry milling techniques. Successful work in preparing stable nanosuspensions using dry-grinding of poorly soluble drugs with soluble polymers and copolymers after dispersing in a liquid media has been reported [42]. Itoh *et al* [35] reported the colloidal particles formation of many poorly water soluble drugs; griseofulvin, glibenclamide and nifedipine obtained by grinding with polyvinylpyrrolidone (PVP) and sodium dodecylsulfate (SDS).

Many soluble polymers and co-polymers such as PVP, polyethylene glycol (PEG), hydroxypropyl methylcellulose (HPMC) and cyclodextrin derivatives have been used [43]. Physicochemical properties and dissolution of poorly water soluble drugs were improved by co-grinding because of an improvement in the surface polarity and transformation from a crystalline to an amorphous drug [44]. Dry co-grinding can be carried out easily and economically and can be conducted without organic solvents. The co-grinding technique can reduce particles to the submicron level and a stable. Table 3 shows some drugs and their status in market.

TABLE 3: SOME DRUGS AND THEIR STATUS IN MARKET

Drug	Category	Route of Administration	Status
Fenofibrate	Anticancer	Oral	Phase I
Rapamuane	Antiemetic	Oral	Marketed
Emend	Antiasthamatic	Oral	Marketed
Thymectacin	Antidiabetic	I.V.	Phase I/II
Silver	Eczema, Atopic dermatitis	Topical	Phase I
Busulfan	Hypolidemic	Intrathecal	Phase I
Paclitaxel	Anticancer	I. V.	Phase IV
Insulin	Antidiabetic	Oral	Phase I
Budesonide	Anticancer	Pulmonary	Phase I

Physical, Chemical and Biological Properties of Nanosuspensions: Nanosuspension formulation increases the saturation solubility as well as dissolution rate. Basically the saturation solubility is a compound specific constant which is temperature dependent. The saturation solubility also depends on the polymorphism of the drug as different polymorphs have different solubilities. It is also dependent on the particle size. This size-depend encycomes only into effect for particles having a size below approximately 1 µm. Another marked property is the adhesiveness generally described for nanoparticles [45].

As the particle size decreases the adhesive properties of the particles will be improved and thus improved oral delivery of poorly soluble drugs. Improved bioavailability, improved dose proportionality, reduced fed/fasted variability, reduced inter-subject variability and enhanced absorption rate (both human and animal data) [46] are some of the main effects observed on oral administration. These data have been acquired *in vivo* in animals but also in humans as reported by the company Nano Systems. A drastically remarkable report is that of the increase in bioavailability for danazole from 5 % (as macrosuspension) to 82% (as nanosuspension) [46]. The application of high

pressures during the production of nanosuspensions was found to promote the amorphous state [47]. The degree of particle fineness and the fraction of amorphous particles in the nanosuspensions were found to be dependent on production pressure number of cycles of homogenisation and hardness of drug. The increase in the amorphous fraction leads to a further increase of the saturation solubility. The homogenization process (giving uniform particle size) was able to overcome Ostwald ripening [48] which means physical long-term stability as an aqueous suspension [49].

In oral drug administration, the bioavailability mainly depends upon the solubility of the drug, highly active compounds have failed in the past because their poor solubility has limited *in vivo* absorption and did not lead to effective therapeutic concentrations. As an example, Atovaquone is given orally three times 750 mg daily, because of the low absorption of only 10–15%. Oral administration of nanosuspensions can overcome this problem because of the high adhesiveness of drug particles sticking on biological surfaces and prolonging the absorption time.

Evaluation of Nanosuspensions [50-51]: The characterisation of the nanosuspensions is also similar to that of the suspensions such as colour, odour, presence of impurities and other important characteristics as mentioned below.

- ***In-Vitro* Evaluations:**
  - **Particle size and size distribution**
  - **Particle charge (Zeta Potential)**
  - **Crystalline state and morphology**
  - **Saturation solubility and dissolution velocity**
  - **Stability**
- ***In-vivo* evaluation:**
- ***In-Vitro* Evaluations:**

- **Particle size and size distribution:** It is the most important parameter in the evaluation of the suspensions as it is having the direct effect on the solubility and dissolution rate and the physical stability of the formulation. The mean particle size and the width of particle size can be determined by Photon Correlation Spectroscopy (PCS) [52], laser diffraction and coulter current multisizer. Particle size and polydispersity index (PI) governs the saturation solubility, dissolution velocity and biological performance. PCS measures the particle size in the range of 3nm-3 µm only. PI governs the physical stability of nanosuspension and should be as low as possible for long-term stability (Should be close to zero). LD measures volume size distribution and measures particles ranging from 0.05- 80µm upto 2000µm. Atomic Force Microscopy is used for visualization of particle shape [53]. For IV use, particles should be less than 5 µm, considering that the smallest size of the capillaries is 5-6 µm and hence a higher particle size can lead to capillary blockade and embolism.

- **Particle charge (Zeta Potential):** The particle charge is of importance in the study of the stability of the suspensions. Usually the zeta potential of more than ±40mV will be considered to be required for the stabilisation of the dispersions. For electrostatically stabilized nanosuspension a minimum zeta potential of ±30mV is required and in case of combined steric and electrostatic stabilization it should be a minimum of ±20mV of zeta potential is required.
- **Crystalline Sate and Particle Morphology:** It is of importance as there are chances of the polymorphism during the storage of the nanosuspensions. Hence it is necessary to study the crystal morphology of the drug in suspension. Differential Scanning Calorimetry

(DSC) is most commonly used for such studies [54]. When nanosuspensions are prepared drug particles may get converted to amorphous form hence it is essential to measure the extent of amorphous drug generated during the production of nanosuspensions. The X-Ray Diffraction (XRD) is commonly used for determining change in crystallinity and the extent of the amorphous form of drug [55].

- **Saturation solubility and Dissolution Velocity:** The main advantage associated with the nanosuspensions is improved saturation solubility as well as dissolution velocity. These are studied in different physiological solutions at different pH. Kelvin equation and the Ostwald-Freundlich equations can explain increase in saturation solubility. Determination of these parameters is useful to assess *in vivo* performance of the formulation.

- **Stability of Nanosuspensions:** Stability of the suspensions is dependent on the particle size. As the particle size reduces to the nanosize the surface energy of the particles will be increased and they tend to agglomerate. So stabilizers are used which will decrease the chances of Ostwald ripening and improving the stability of the suspension by providing a steric or ionic barrier. Typical examples of stabilizers used in nanosuspensions are cellulosics, poloxamer, polysorbates, lecithin, polyoleate and povidones. Lecithin may be preferred in developing parenteral nanosuspensions [40].
- ***In vivo* evaluation:** The *in vivo* evaluation of the nanosuspensions is specific to drug and route of administration. Most commonly the formulation was given by required route of administration and the plasma drug levels were estimated using HPLC-UV visible Spectrophotometry. Other parameters which are generally evaluated *in vivo* are
  - Surface hydrophilicity/hydrophobicity (determines interaction with cells prior to phagocytosis)
  - Adhesion properties
  - Interaction with body proteins

APPLICATIONS: Formulating the drug as nanosuspensions increases the saturable concentration, dissolution rate as well as bioavailability of the drug. These nanosuspensions are having application in different routes of administrations like oral, parenteral, topical, ophthalmic, mucoadhesive, pulmonary and targeted drug delivery. Oral administration of nanosuspensions is a drug delivery strategy, not only to improve bioavailability, but also to target gastrointestinal bacterial and parasitic infections because of improved adhesion properties. Nanosuspension technology is considered as suitable new colon delivery systems for the treatment of colon cancer, helminth infections, gastrointestinal inflammation or GIT associated diseases like sprue (zoeliaki).

Infections like tuberculosis, listeriosis, leishmaniasis, and toxoplasmosis are caused by parasites residing the macrophages of the MPS, thus being relatively easily accessible by I.V. injected particles. The I.V. injected particles are heavily and quickly taken up by the MPS cells in case they absorb uptake promoting proteins like apolipoproteins. However, some parasites do also reside in the brain (CNS). The brain-localized parasite mostly leads to relapsing infections if not cured. Therefore, it would be of importance to target drug nanoparticles via surface modification to the brain. A successful targeting of the peptide, dalargin, to the brain using Tween 80® surface modified polyisobutylcyanoacrylates nanoparticles has been reported by Kreuter et al. [56]. A nanosuspension of Amphotericin B developed by Kayser *et al.* showed a significant improvement in its oral absorption in comparison with the

conventional commercial formulations [57]. In case of I.V administration the particle size less than 5µm is preferred. The particle size in nano range will favour the passage of the drug particles into the small capillaries in the body without any blockade. A stable intravenously injectable formulation of omeprazole has been prepared to prevent the degradation of orally administered omeprazole [37].

Aqueous suspensions of the drug can be easily nebulised and given by pulmonary route as the particle size is very less. Different types of nebulisers are available for the administration of liquid formulations. Some of the drugs successfully tried with pulmonary route are budesonide, ketotifen, ibuprofen, indomethacin, nifedipine, itraconazole, interleukin-2, p53 gene, leuprolide, doxorubicin etc. [58] Nanosuspensions can be used for targeted delivery also as the surface of the particle can be suitably modified to make them target specific. Kayser formulated a nanosuspension of Aphidicolin to improve drug targeting against leishmania-infected macrophages [26]. Scholer et al. Prapeared a nanosuspension formulation of Atovaquone and showed an improved drug targeting to the brain in the treatment of toxoplasmic encephalitis in a new murine model infected with Toxoplasma gondii [55].

CONCLUSIONS: Nanosuspensions are chiefly seen as vehicles for administering poorly water soluble drugs have been largely solved the dissolution problems to improve drug absorption and bioavailability. Nanosuspension technology can be combined with traditional dosage forms: tablets, capsules, pellets, and can be used for parenteral products. They have recently received increasing attention as colloidal carriers for targeted delivery of various anticancer drugs, photosensitizers, neutron capture therapy agents or diagnostic agents. Because of their submicron size they are easily targeted to the tumour area. Moreover the possibility of surface functionalization with a targeting moiety has open new avenues for targeted delivery of drugs, genes, photosensitizers and other molecules to the desired area. To take advantage of nanosuspension drug delivery, simple formation technologies and variety applications, nanosuspensions will continue to be of interest as oral formulations and non-oral administration develop in the future. It is expected that future research and development work will be carried out in the near future for clinical realization of these targeted delivery vehicle.

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Physical and chemical stability of drug nanoparticles ☆

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ABSTRACT

As nano-sizing is becoming a more common approach for pharmaceutical product development, researchers are taking advantage of the unique inherent properties of nanoparticles for a wide variety of applications. This article reviews the physical and chemical stability of drug nanoparticles, including their mechanisms and corresponding characterization techniques. A few common strategies to overcome stability issues are also discussed.

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Contents

1. Introduction

With significant attention focused on nanoscience and nanotechnology in recent years, nanomaterial-based drug delivery has been propelled to the forefront by researchers from both academia and industry [1–3]. Various nano-structured materials were produced and applied to drug delivery such as nanoparticles [4], nanocapsules [5],

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nanotubes [6], micelles [7], microemulsions [8] and liposomes [9]. In general, the term "nanoparticles" refers to particles with sizes between 1 and 100 nm. However, submicron particles are also commonly referred as nanoparticles in the field of pharmaceutics and medicine [10–14]. Nanoparticles are categorized as nanocrystals [10], polymeric [15], liposomal [9] and solid lipid nanoparticles (SLN) [16] depending on their composition, function and morphology. Given the extensive available literature reviews on SLN, polymeric and liposomal nanoparticles [4,9,15–18], this article will focus only on nanocrystals (pure drug nanoparticles).

The unique nano-scale structure of nanoparticles provides significant increases in surface area to volume ratio which results in notably different behavior, both *in-vitro* and *in-vivo*, as compared to the traditional microparticles [10–12]. Consequently, drug nanocrystals have been extensively used in a variety of dosage forms for different purposes [10,11,14,19,20], such as improving the oral bioavailability of poorly water-soluble drugs by utilizing enhanced solubility and dissolution rate of nanoparticles [21–23]. In the field of pulmonary drug delivery, the nanoparticles are able to deliver the drugs into the deep lungs, which is of great importance for systemically absorbed drugs [11,14]. In addition, injection of poorly water-soluble nanosuspension drugs is an emerging and rapidly growing field that has drawn increasing attention due to its benefits in reducing toxicity and increasing drug efficacy through elimination of co-solvent in the formulation [10,20].

Despite the advantages of drug nanocrystals, they present various drawbacks including complex manufacturing [12,24–26], nanotoxicity [27] and stability issues [10,19,20]. Stability is one of the critical aspects in ensuring safety and efficacy of drug products. In intravenously administered nanosuspensions, for example, formation of larger particles (>5 μm) could lead to capillary blockade and embolism [20], and thus drug particle size and size distribution needs to be closely monitored during storage. The stability issues of drug nanoparticles could arise during manufacturing, storage and shipping. For instance, the high pressure or temperature produced during manufacturing can cause crystallinity change to the drug particles [12,26,28]. Storage and shipping of the drug products may also bring about a variety of stability problems such as sedimentation, agglomeration and crystal growth [29–31]. Therefore, stability issues associated with drug nanocrystals deserve significant attention during pharmaceutical product development. This article reviews existing literature on drug nanoparticle stability, including theory/mechanisms, methods used to tackle the stability problems and characterization techniques, and provides recommendations to improve the current practices. Since the stability issues related to nanoparticle dry powders are usually trivial, this review will only focus on stability of nanosuspensions (drug nanoparticles dispersed in a liquid medium).

2. Stability of drug nanoparticles

2.1. Effect of dosage form on stability

The unique characteristics of drug nanoparticles have enabled their extensive application in various dosage forms including oral, parenteral, ocular, pulmonary, dermal and other specialized delivery systems [10,11,13,20,32]. Although different dosage forms may share some common stability issues, such as sedimentation, particle agglomeration or crystal growth, their effects on drug products are quite different. For instance, particle agglomeration could be a major issue in pulmonary drug delivery since it affects deposition amount/site, and thus drug efficacy. On the other hand, agglomeration in intravenous formulations can cause blood capillary blockage and obstruct blood flow. Moreover, the selection of stabilizers is also closely related to dispersion medium, dosage form and strictly governed by FDA regulations. To date, there is a wide variety of choices on the approved stabilizers for oral dosage form whereas the excipients allowed for inhalation are very limited [33].

Drug nanoparticles exist in the final drug products either in dry powder or suspension form. Examples of the dry powder form include the dry powder inhaler, lyophililized powder for injection and oral tablets or capsules. Solid dosage forms usually have good storage stability profiles, which is why a common strategy to enhance nanosuspension stability is to transform the suspension into solid form [19,25]. Most of the reported stability concerns arise from nanosuspensions in which the drug nanoparticles are dispersed in a medium with or without stabilizers. In addition, mechanisms involved in the stability of small and large biomolecule formulations are different due to their molecular structure differences. A small molecule drug is defined as a low molecular weight non-polymeric organic compound while large biomolecules refer to large bioactive molecules such as protein/peptide. One of the major issues with protein/peptide stability is to maintain the 3-dimensional molecular conformation, such as secondary and tertiary structure in order to keep their biological activities [34,35], whereas there is no such concern for small organic molecules.

2.2. General stability issues related to nanosuspensions

Stability issues associated with nanosuspensions have been widely investigated and can be categorized as physical and chemical stability. The common physical stability issues include sedimentation/creaming, agglomeration, crystal growth and change of crystallinity state.

2.2.1. Sedimentation or creaming

Drug particles can either settle down or cream up in the formulation medium depending on their density relative to the medium. The sedimentation rate is described by Stokes' law [36,37] which indicates the important role of particle size, medium viscosity and density difference between medium and dispersed phase in determining the sedimentation rate. Decreasing particle size is the most common strategy used to reduce particle settling. Matching drug particles density with medium or increasing medium viscosity are the other widely used approaches to alleviate sedimentation problems [37,38]. Fig. 1 shows different sedimentation types that occur in suspension formulations.

In a deflocculated suspension (Fig. 1a), particles settle independently as small size entities resulting in a slow sedimentation rate. However, densely packed sediment, known as caking [39], is usually formed due to the pressure applied on each individual particle. This sediment is very difficult to be re-dispersed by agitation [36,37,39] and would be detrimental to the drug products performance. In the flocculated suspension (Fig. 1b), the agglomerated particles settle as loose aggregates instead of as individual particles [36,37]. The loose aggregates have a larger size compared to the single particle, and thus higher sedimentation rate. The loose structure of the rapidly settling flocs contains a significant amount of entrapped medium and this structure is preserved in the sediment. The final flocculation volume is therefore relatively large and the flocs can be easily broken and re-dispersed by simple agitation. K.P. Johnston et al. [40,41] have recently attempted to achieve stable nanosuspensions via a novel design of flocs structure called "open flocs", as illustrated in Fig. 1c. Thin film freezing was used to produce BSA nanorods with aspect ratio of approximately 24. These BSA nanorods were found to be highly stable when dispersed into hydrofluoroalkane (HFA) propellant, with no apparent sedimentation observed for 1 year. Due to the high aspect ratio of BSA nanorods and relatively strong attractive Van der Waals (VDW) forces at the contact sites between the particles, primary nanorods were locked together rapidly as an open structure upon addition of HFA, inhibiting collapse of the flocs [41]. The low-density open flocs structure was then filled with liquid HFA medium, preventing particle settling. Similar results were shown using needle

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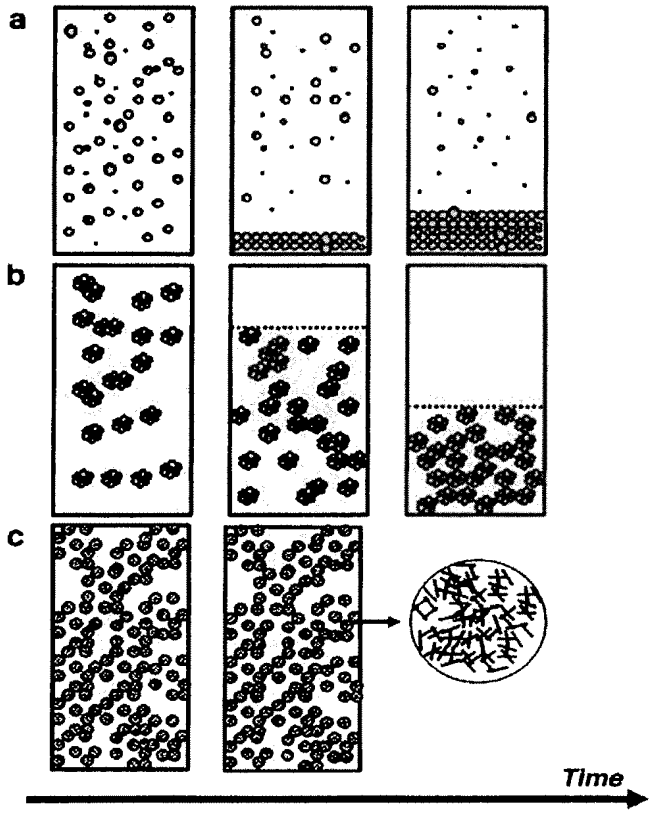

Fig. 1. Sedimentation in (a) deflocculated suspension; (b) flocculated suspension; and (c) open flocs-based suspension.

and plate shaped itraconazole nanoparticles with aspect ratios between 5 and 10 [40].

Although sedimentation is one of the key issues for colloidal suspension, the reported studies examining sedimentation issues in aqueous-based nanosuspensions are very scarce. This could be due to (i) surfactants are generally used in most of the nanosuspensions to inhibit particle agglomeration in the medium, which alleviates the sedimentation issues and (ii) the small nano-sized particles significantly reduce the sedimentation rate. In addition, many of the aqueous nanosuspensions are transformed to dry solid form by spray drying or freeze drying to circumvent the long-term sedimentation issue. Unfortunately, this solidification process cannot be applied to non-aqueous nanosuspensions where sedimentation/creaming is commonly present. An example to illustrate this is metered dose inhaler (MDI) formulations where the nanoparticles are suspended in HFA propellants. Sedimentation or creaming is a key aspect affecting stability of these formulations. Particle engineering to optimize particle surface properties and morphology, e.g. hollow porous particles [42], and introduction of surfactant(s) is generally employed to alleviate the issue.

2.2.2. Agglomeration

The large surface area of nanoparticles creates high total surface energy, which is thermodynamically unfavorable. Accordingly, the particles tend to agglomerate to minimize the surface energy. Agglomeration can cause a variety of issues for nanosuspensions including rapid settling/creaming, crystal growth and inconsistent dosing. The most common strategy to tackle this issue is to introduce stabilizers to the formulation. In addition to safety and regulation considerations, selection of stabilizers is based on their ability to provide wetting to surface of the particles and offer a barrier to prevent nanoparticles from agglomeration [13,19].

There are two main mechanisms through which colloidal suspensions can be stabilized in both aqueous and non-aqueous medium, i.e. electrostatic repulsion and steric stabilization [10,36,37]. These two mechanisms can be achieved by adding ionic and non-ionic stabilizers into the medium, respectively. Stabilization from electrostatic repulsion can be described by the classic Derjaguin–Landau–Verwey–Overbeek (DLVO) theory [43,44]. This theory mainly applies to aqueous suspension while its application in non-aqueous medium is still not well-understood [33]. The DLVO theory assumes that the forces acting on the colloidal particles in a medium include repulsive electrostatic forces and attractive VDW forces. The repulsive forces are originated from the overlapping of electrical double layer (EDL) surrounding the particles in the medium, and thus preventing colloidal agglomeration. The EDL consist of two layers: (i) stern layer composed of counter ion attracted toward the particle surface to maintain electrical neutrality of the system and (ii) Gouy layer which is essentially a diffusion layer of ions (Fig. 2).

The total potential energy (V_T) of particle–particle interaction is a sum of repulsion potential (V_R) generated from electric double layers and attraction potential (V_A) from the VDW forces. V_A is determined by the Hamaker constant, particle size and inter-particulate distance while V_R depends on particle size, distance between the particles, zeta potential, ion concentration and dielectric constant of the medium. V_R is extremely sensitive to ion concentration in the medium. As the ion strength is increased in the medium, the thickness of EDL decreases due to screening of the surface charge [36,37]. This causes decrease in V_R, increasing the susceptibility of the dispersed particles to form aggregates. Zeta potential (ZP) is electric potential at the shear plane which is the boundary of the surrounding liquid layer attached to the moving particles in the medium. ZP is a key parameter widely used to predict suspension stability. The higher the ZP, the more stable the suspension is.

In the case of steric stabilization, amphiphilic non-ionic stabilizers are usually utilized to provide steric stabilization which is dominated by solvation effect. As the non-ionic stabilizers are introduced into nanosuspensions, they are absorbed onto the drug particles through an anchor segment that strongly interacts with the dispersed particles, while the other well-solvated tail segment extends into the bulk medium (Fig. 3).

As two colloidal particles approach each other, the stabilizing segments may interpenetrate, squeezing the bulk medium molecules out of the inter-particulate space as illustrated in Fig. 3. This interpenetration is thermodynamically disfavored when a good solvent is used as the bulk medium to stabilize the tail [36]. Accordingly, provided that the stabilizers can be absorbed onto the particle surface through the anchor segment, strong enthalpic interaction (good solvation) between the solvent and the stabilizing segment of the stabilizer is the key factor to achieve steric stabilization and prevent particles from agglomeration in the medium [36,37]. In addition to solvation, the stabilizing moiety needs to be sufficiently long and dense to maintain a steric barrier that is capable of minimizing particle–particle interaction to a level that the VDW attractive forces are less than the repulsive steric forces [43–45].

The main drawback associated with the steric stabilization is the constant need to tailor the anchoring tail according to the particular drug of interest. Due to the lack of fundamental understanding of interaction between the stabilizers and dispersed nanoparticles, current surfactant screening approaches to achieve a successful steric stabilization are mostly empirical and could be very burdensome [45–49]. In addition, the solvation of the stabilizing segment is susceptible to variation in temperature. Stabilizer concentration could also play a role in causing suspension instability by affecting the absorption affinity of non-ionic stabilizers to drug particles surface. Deng et al.

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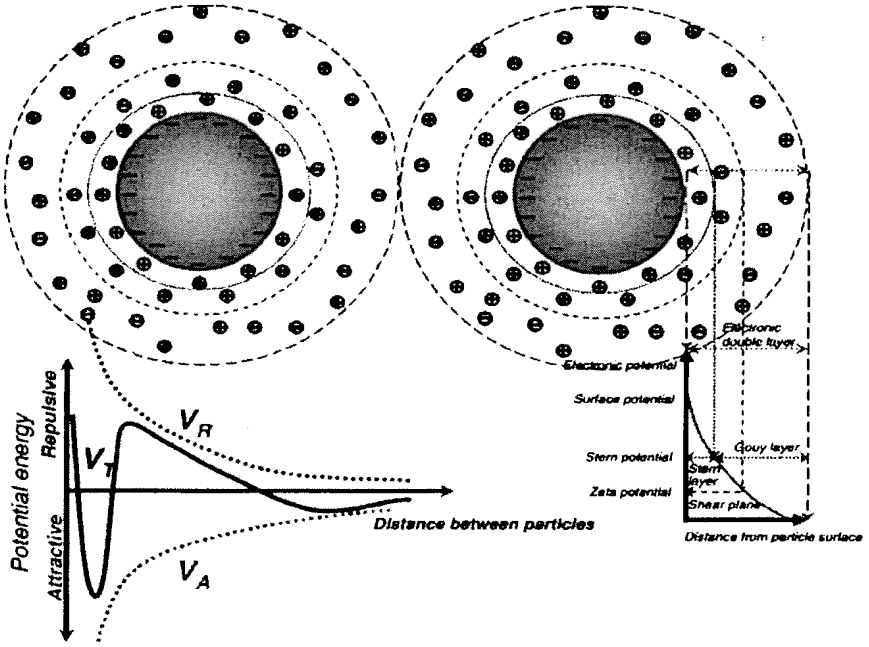

Fig. 2. Illustration of classical DLVO theory. Attractive forces are dominant at very small and large distances, leading to primary and secondary minimum, while repulsive forces are prevailing at intermediate distances and create net repulsion between the dispersed particles, thus preventing particle agglomeration.

[50] used Pluronic® F127 to stabilize paclitaxel nanosuspensions. It was reported that stabilizers had high affinity to nanocrystals surface at concentrations below critical micelle concentration (CMC), and increasing concentrations above CMC caused loss of F127 affinity to the nanocrystals and thus unstable formulation. This was because F127 monomers on the nanocrystals surface started to aggregate with each other to form micelles as the concentration was increased to the CMC level, leading to a lower affinity to the drug crystals. Temperature was also shown to affect the stabilizer affinity to drug crystals. This is expected since the CMC level is dependent on temperature.

It is apparent that combination of the two stabilization mechanisms can be very beneficial in achieving a stable colloidal dispersion. In addition, the combination of a non-ionic stabilizer with an ionic stabilizer reduces the self repulsion between the ionic surfactant molecules, leading to closer packing of the stabilizer molecules [10,51].

Besides the steric and electrostatic stabilization mechanisms, some other stabilization mechanisms have also been reported. Makhlof et al. produced indomethacin (IMC) nanocrystals using the emulsion solvent diffusion technique [52]. The nanoparticles were stabilized using various cyclodextrins (CyDs) without adding any surfactants. The stabilizing effect was attributed to the formation of a CyD network in the aqueous medium via intermolecular interaction of CyD molecules. The network-like structure was believed to prevent aggregation and crystal growth of IMC nanoparticles initially produced from the solvent diffusion process. Similar stabilization mechanism was also observed in another study where budesonide microsuspension was stabilized with hydroxypropyl-beta-cyclodextrin in HFA medium [53]. Another approach to enhance suspension stability that has increasingly been utilized is engineering of particle morphology. One breakthrough in this area was the porous particle

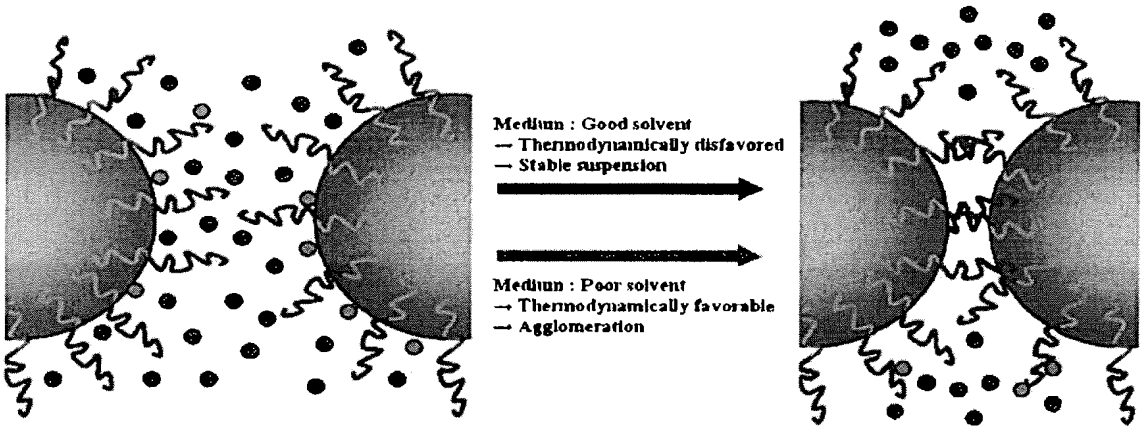

Fig. 3. Steric stabilization mechanisms according to Gibbs free energy: $\Delta G = \Delta H - T\Delta S$. A positive ΔG indicates stable suspension while negative ΔG induces particle agglomeration. If the medium is a good solvent for the stabilizing moiety, the adsorbed stabilizing layers on the dispersed particles cannot interpenetrate each other when the particles collide. This reduces the number of configurations available to the adsorbed stabilizing tails, resulting in a negative entropy change and positive ΔG. On the other hand, if the dispersion medium is a poor solvent, the adsorbed layers on the particles may interpenetrate thermodynamically and induces particles agglomeration.

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concept that was first introduced by Edwards et al. [54]. The porous particles include hollow porous particle [42] and porous nanoparticle-aggregate particles (PNAPs) [14]. Unfortunately, most of the work has been focused on microsuspension or polymeric colloidal formulations and has not been applied to pure drug nanoparticles.

Table 1 summarizes a few published studies on pharmaceutical nanosuspensions. Due to the vast amount of literature work on the pharmaceutical nanosuspensions, this review will focus only on the studies that provide a more profound enlightenment on the stabilizer selection for nanosuspensions. The summary table shows that most of nanosuspensions were generated in aqueous medium, with only a limited number of nanosuspensions made in non-aqueous environment. The commonly used ionic stabilizers in aqueous medium include sodium dodecyl sulfate (SDS), sodium lauryl sulfate (SLS), lecithin and docusate sodium. The non-ionic surfactants used in aqueous medium are usually selected from Pluronic® surfactants, Tween 80, polyethylene glycol (PEG), polyvinyl alcohol (PVA) polyvinylpyrrolidone (PVP) and cellulose polymers such as hydroxypropyl cellulose (HPC) and hydroxypropyl methylcellulose (HPMC).

The stabilizers are not only used to provide short- and long-term storage stability for nanosuspensions, but also to achieve successful formation and stabilization of nanocrystals during particle production. Lee et al. designed and synthesized various amino acid copolymers containing lysine as the hydrophilic segments with alanine, phenylalanine or leucine as hydrophobic moieties [49]. Wet comminution was used to produce naproxen nanosuspensions in presence of HPC and amino acid copolymers. Lysine copolymer with alanine was unable to produce submicron particles while the other copolymers with phenylalanine and leucine were capable of forming the nanoparticles. The size of nanocrystals was proven to be constant over 1 month storage and the crystallinity was also shown to be preserved after the wet comminution process. Furthermore, hydrophobicity of the copolymers was identified as the key factor in achieving the stable nanosuspensions, attributed to strong polymer adsorption onto the hydrophobic drug surfaces. Although this work did not provide an in-depth discussion on how the copolymers interacted with the drug nanoparticles, it illustrated the importance of careful selection of the anchor group (that is attached to the drug surface) in facilitating the production of a stable nanosuspesion. In the subsequent study [45], they attempted to understand the nature of interactions between polymeric stabilizers and drugs with different surface energies. Nanocrystals of seven model drugs with PVP K30 and HPC as stabilizers were generated using wet comminution. It was expected that a close match of surface energy between the stabilizers and drug crystals would promote the absorption of stabilizers onto drug particles, and thus help in reducing the particle size during the wet comminution process. Although surface energy did not seem to correlate well with particle size for HPC stabilized system, some trend was observed for PVP stabilized suspension with only one exception.

A further study with seven stabilizers (non-ionic stabilizers: HPC, PVP K30, Pluronic® F127 & F68, PEG and ionic stabilizers: SDS and benzethoinum chloride) and eleven model drugs was conducted by the same group in order to provide more understanding on the stabilization mechanism [48]. Again, the general trend between surface energy and particle size reduction was not observed in this work. PEG was unsuccessful in reducing the particle size of most drug candidates while the other non-ionic stabilizers proved to be effective in reducing the size of five drug candidates that had similar surface energies to the stabilizers. F68 was shown to be the most effective stabilizer (successfully stabilizing nine drug candidates), which could be due to its strong chain adsorption onto the drug crystals through the hydrophobic polypropylene glycol (PPG) units. F127 was found to be less efficient than F68 likely because the short processing time led to inefficient physical adsorption of higher molecular weight F127 to the drug surface. This study demonstrated that a combination of ionic and non-ionic stabilizers is not always beneficial to enhance stabilization. A few combinations of SDS or benzethoinum chloride with various non-ionic stabilizers resulted in positive stability effects while the others did not. The effects of physicochemical properties of the drugs on the stabilization were also explored in this study. In general, drugs with lower aqueous solubility, higher molecular weight and higher melting point were shown to have higher chance for successful nanosuspension formation.

Van Eerdenbrugh et al. conducted an expanded study using 13 stabilizers at 3 different concentrations to stabilize 9 drug compounds [47]. The particles were generated using the wet milling technique. The success rate in producing nanosuspensions using polysaccharide based stabilizers [HPMC, methylcellulose (MC), hydroxyethylcellulose (HEC), HPC, carboxymethylcellulose sodium (NaCMC), alginic acid sodium (NaAlg)] was limited by the high viscosity of these polymeric stabilizer solutions. Increasing concentration of these stabilizers did not appear to be helpful. In contrast, the other stabilizers [PVP K30, PVP K90, PVA, Pluronic® F68, polyvinyl alcohol–polyethylene glycol graft copolymer (K-IR), Tween 80 and D-α-tocopherol polyethylene glycol 1000 succinate (TPGS)] did not encounter the viscosity issue. PVA was ineffective in producing the nanosuspension and the success probability of PVP K30, PVP K90, F68 and K-IR is highly dependent on their concentration. Higher concentrations (25 wt.% and 100 wt.%) increased the stabilizing efficacy significantly. Tween 80 and TGPS were proven to be most effective stabilizers. Addition of TGPS (at concentrations >25 wt.%) allowed nanosuspension formation for all tested drug compounds. No correlation was observed between drug physicochemical properties (molecular weight, melting point, log p, solubility and density) and nanosuspension formation success rate. It was demonstrated that surface hydrophobicity of the drug candidates was the driving force for nanoparticles agglomeration, thus lowering the success rate of nanosuspension production.

Mishra et al. explored nanosuspension stability issues during both production and storage [29]. Hesperetin nanosuspensions were produced using HPH with Pluronic® F68, alkyl polyglycoside (Plantacare 2000) and inulin lauryl carbamate (Inutec SP1), or Tween 80 as stabilizers. It was demonstrated that all stabilizers were suitable for successful production of hesperetin nanosuspensions. The size of nanocrystals was dependent on power density applied in the homogenization process and the hardness of the crystals. The effect of stabilizers on the particle size was negligible. Short-term stability over a period of 30 days was examined in order to evaluate the stabilizer efficiency. ZP was measured as a key parameter to predict the stability. In distilled water, the ZP values of all the nanosuspensions fell between −30 and −50 mV and the values dropped significantly in the original dispersion medium. This can be explained by the fact that adsorbed layers of large molecules shifted the shear plane to a longer distance from the particle surface, thus reducing the measured value of zeta potential (Fig. 4). However, the low ZP value does not point to an unstable suspension in this case, which could be due to the additional presence of steric stabilization mechanism. Both Inutec and Plantacare stabilized nanosuspensions also showed significant reduction of ZP measured from water to dispersion medium, indicating a thick absorbed steric layer and good stability. F68 exhibited only slight decrease in ZP, indicating a relatively thin stabilization layer. The ZP value of Tween 80 was only −13 mv in the dispersion medium, pointing to a potentially problematic stabilization. The study demonstrated that zeta potential measurement is a good predictor for storage stability. Nanosuspensions stabilized by Inutec and Plantacare were stable at all storage conditions (4, 25 and 40 °C) up to 30 days while F68 stabilized nanosuspensions were shown to be less stable. The Tween 80 formulation stability was the poorest. Pardeike et al. [30] conducted a similar study using phospholipase A2 inhibitor PX-18 nanosuspensions produced by HPH with Tween 80 as stabilizer. In this work, ZP of the homogenized nanosuspensions was dropped from −50 mV to

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t1.1 **Table 1**
Literature summary of pharmaceutical nanosuspensions.

t1.2

t1.3 Nanoparticles compound	Manufacturing technique	Delivery route	Dispersion medium	Stabilizers	Reference
t1.4 Oridonin	HPH	NA	Water	PVP K25, Brij 78, SDS, Pluronic® F68, lecithin	Gao et al. (2007) [55]
t1.5 Oridonin	HPH	IV	Water	Pluronic® F68, lecithin	Gao et al. (2008) [56]
t1.6 Budesonide	HPH	Inhalation	Water	Lecithin, Span 85, tyloxapol, cetyl alcohol	Jacobs et al. (2002) [57]
t1.7 Buparvaquone	HPH	Inhalation	Water	Pluronic® F68 and PVA	Hernadez-Trejo et al.(2005) [58]
t1.8 Buparvaquone	HPH	Oral	Water	Pluronic® F68 and lecithin	Jacobs et al. (2002) [59]
t1.9 Diclofenac acid	HPH	Oral	Water	Pluronic® F68	Lai et al. (2009) [60]
t1.10 Azothromycin	HPH	NA	Water	Lecithin, Pluronic® F68, Tween 80	Zhang et al. (2007) [61]
t1.11 Rutin	HPH	Oral	Water	SDS	Mauludin et al. (2009) [62]
t1.12 Rutin	HPH	Oral	Water	SDS, Tween 80, Pluronic® F68, PVA	Mauludin et al. (2009) [63]
t1.13 Tarazepide	HPH	NA	Water	Tween 80, Pluronic® F68	Jacobs et al. (2000) [64]
t1.14 Omeprazole	HPH	IV	Water	Pluronic® F68	Moschwitzer, (2004) [65]
t1.15 Amphotericin B	HPH	Oral	Water	Tween 80, Pluronic® F68	Kayser et al. (2003) [22]
t1.16 Nimodipine	HPH	IV	Water	Pluronic® F68, sodium cholic acid and mannitol	Xiong et al., (2008) [66]
t1.17 Albendazole	HPH	Oral	Water	SLS, Carbopol, PS 80, hpmc	Kumar et al. (2008) [23]
t1.18 RMKP 22	HPH	NA	Water	Phospholipon 90	Peters et al. (1999) [67]
t1.19 Hesperetin	HPH	Dermal	Water	Pluronic® F68, Inutec SP1, Tween 80 and Plantacare 2000	Mishra et al. (2009) [29]
t1.20 Hydrocortisone, prednisolone and dexamethasone	HPH	Opthalmic	Water	Pluronic® F68	Kassem et al. (2007) [68]
t1.21 Ascorbyl palmitate	HPH	NA	Water	SDS, Tween 80	Teeranachaideekul et (2008) [69]
t1.22 RMKK99	HPH	NA	Water	Potassium oleate, Tween 80	Krause et al. (2001) [70]
t1.23 Nifedipine	HPH	NA	Water	HPMC	Hecq et al. (2005) [71]
t1.24 Undisclosed	HPH	Oral	Water	SLS, HPMC, PVA, Acaciae Gum, Pluronic® F127	Hecq et al. (2006) [72]
t1.25 Hydroxycamptothecin	HPH	NA	Water	Lipoid S75, Pluronic® F68, Solutol® HS 15	Zhao et al. (2010) [73]
t1.26 Asulacrine	HPH	IV	Water	Pluronic® F68	Ganta et al. (2009) [74]
t1.27 RMKP 22	HPH	NA	Water	Tween 80	Muller et al. (1998) [75]
t1.28 RMKP 22	HPH	NA	Water	Tween 80, Glycerol	Grau et al. (2000) [76]
t1.29 PX-18	HPH	NA	Water	Tween 80	Pardeike et al. (2010) [30]
t1.30 PX-18	HPH	NA	Water	Tween 80	Wang et al. (2010) [77]
t1.31 Silybin	HPH	Oral, IV	Water	Lecithin, Poloxamer 188	Wang et al. (2010) [78]
t1.32 Tarazepide	HPH	IV	Water	Pluronic® F68, Tween 80, Glycerol	Jacobs et al. (2000) [64]
t1.33 Omeprazole, albendazole and danazol	Wet milling	Oral	Water	Pluronic® F108, F68	Tanaka et al. (2009) [79]
t1.34 Fluticasone, budesonide	Wet milling	Inhalation	Water	Tween 80	Yang et al. (2008) [80]
t1.35 Naproxen	Wet milling	NA	Water	HPC, arginie hydrochloride	Ain-Ai et al. (2008) [81]
t1.36 Loviride	Wet milling	NA	Water	Tween 80, Pluronic® F68	Van Eerdenbrugh et al. (2007) [82]
t1.37 Nine different compounds	Wet milling	NA	Water	13 different stabilizers	Van Eerdenbrugh et al. (2009) [47]
t1.38 Zinc Insulin	Wet milling	NA	Water	Pluronic® F68, sodium deoxycholate	Merisko-Liversidge et al. (2004) [83]
t1.39 Ethyl Diatrizoate	Wet milling	NA	Water	Poloxamine 908	Na et al. (1999) [84]
t1.40 Cinnarizine, itraconazole and phenylbutazone	Wet milling	NA	Water	TPGS 1000	Van Eerdenbrugh et al. (2008) [85]
t1.41 Nine different compounds	Wet milling	NA	Water	TPGS 1000	Van Eerdenbrugh et al. (2008) [86]
t1.42 Beclomethasone dipropionate	Wet milling	Inhalation	Water	PVA	Wiedmann et al. (1997) [87]
t1.43 Rilpivirine	Wet milling	Parenteral	Water	Pluronic® F108, TPGS 1000	Baert et al. (2009) [88]
t1.44 Undisclosed	Wet milling	NA	Water	Plasdone S-630, docusate sodium	Deng et al. (2008) [89]
t1.45 Piposulfan, etoposide, camptothecin, paclitaxel	Wet milling	NA	Water	Tween 80, Span 80, Pluronic® F108, F127	Merisko-Liversidge et al. (1996) [90]
t1.46 Naproxen	Wet comminution	NA	Water	Copolymers of amino acids	Lee et al. (2005) [49]
t1.47 Seven different compounds	Wet comminution	NA	Water	HPC, PVP	Choi et al. (2005) [45]
t1.48 Eleven different compounds	Wet comminution	NA	Water	HPC, PVP, PEG, SDS, Pluronic® F68, F127, benzethonium chloride	Lee et al. (2008) [48]
t1.49 Dihydroartemisinin	Vibrational rod milling	NA	Water	PVP K30, sodium deoxycholate	Chingunpitak et al.(2008) [91]
t1.50 Probucol	Vibrational rod milling	NA	Water	PVP, SDS	Pongpeerapat et al. (2008) [92]
t1.51 Ibuprofen	Precipitation, microfluidization	NA	Water	SLS, PVP K30, Pluronic® F68, F127, Tween 80, HPMC	Verma et al. (2009) [31]
t1.52 Hydrocortisone	Precipitation, microfluidization	NA	Water	PVP, HPMC, SLS	Ali et al. (2009) [93]
t1.53 Ibuprofen	Solvent diffusion, melt emulsification	NA	Water	PVA, PVP K25, Pluronic® F68, Tween 80,	Kocbek et al. (2006) [94]
t1.54 Alendronate-gallium, alendronate-gadolinium	Complex precipitation	NA	Water	None	Epstein et al. (2007) [95]
t1.55 Paclitaxel	Stabilization of nanocrystal (SNC)	NA	Water	Pluronic® F127	Deng et al. (2010) [50]
t1.56 Felodipine	Antisolvent precipitation & Wet-milling	NA	Water	PVP K30, SDS, docusate sodium	Lindfors (2007) [96]
t1.57 Naproxen	Antisolvent precipitation	Oral	Water	PVP K15, Pluronic® F127	Chen et al. (2009) [97]
t1.58 Carbamazepine	Antisolvent precipitation	NA	Water	HPMC, PVP K17	Douroumis et al. (2007) [98]
t1.59 Cyclosporin A	Antisolvent precipitation	Inhalation	Water	Tween 80	Tam et al. (2008) [99]
t1.60 Undisclosed	Antisolvent precipitation, Wet milling	IV, Oral	Water	PVP, SDS, Miglyol, docusate sodium	Sigfridsson et al. (2007) [100]
t1.61 β-methasone valerate-17, oxcarbazepine	Antisolvent precipitation	NA	Water	HPMC, lipoid S75, PEG-5 soy sterol	Douroumis et al. (2006) [101]
t1.62 Retinoic acid	Antisolvent precipitation	NA	Water	None	Zhang et al. (2006) [102]

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t1.63 **Table 1** (continued)

	Nanoparticles compound	Manufacturing technique	Delivery route	Dispersion medium	Stabilizers	Reference
t1.65	2-devinyl-2-(1-hexyloxyethyl) pyropheophorbide	Antisolvent precipitation	NA	Water	None	Baba et al. (2007) [103]
t1.66	Nitrendipine	Precipitation–ultrasonication	Oral	Water	PVA	Xia et al. (2010) [104]
t1.67	Indomethancin	Emulsion diffusion	NA	Water	Cyclodextrins	Makhlof et al. (2008) [52]
t1.68	Celecoxib	Emulsion diffusion	Oral	Water	Tween 80, PVP K30, SDS	Dolenc et al. (2009) [105]
t1.69	Griseofulvin	Emulsion diffusion	NA	Water	Tween 80, Oramix CG-110	Trotta et al.(2003) [106]
t1.70	Mitotane	Emulsion diffusion	NA	Water	Tween 80, caprylyl-capryl glucoside, lecithin	Trotta et al.(2001) [107]
t1.71	Griseofulvin	Microemulsion diffusion	NA	Water	Lecithin	Trotta et al.(2003) [108]
t1.72	Lysozyme	Emulsification/freeze-drying	Inhalation	HFA	None	Nyambura et al.(2009) [109]
t1.73	Bovine serum albumin	Thin film freezing	Inhalation	HFA	None	Engstrom et al.(2009) [41]
t1.74	Itraconazole	Thin film freezing	Inhalation	HFA	None	Tam et al.(2010) [40]
t1.75	Insulin	Emulsification + freeze-drying	Inhalation	HFA	Citral, cineole	Nyambura et al.(2009) [110]
t1.76	Salbutamol sulfate	Microemulsion + freeze-drying	Inhalation	HFA	Lecithin, docusate sodium	Dickinson et al. (2001) [111]
t1.77	Salbutamol sulfate	HPH	NA	Acetonitrile	Tween 80	Ahmad et al. (2009) [112]
t1.78	Horseradish peroxidase, carbonic anhydrase, lysozyme, subtilisin carlsberg and α-chymotrypsin	Freeze-drying	NA	Ethyl acetate	Methyl-β-cyclodextrin	Montalvo et al. (2008) [113]
t1.79	Diclofenac	Emulsification + freeze drying	Transdermal	Isopropyl myristate	Sucrose ester	Piao et al. (2007) [114]

around −20 mV when tested from water to dispersion medium. It is generally believed that ZP of ±20 mV is sufficient to maintain a stable formulation with a combined electrostatic and steric stabilization [30]. The PX-18 nanosuspension was shown to be physically stable (no changes in particle size distribution) for more than half year at the storage condition of 5 and 25 °C. However, physical instability was observed after 1 month storage at a higher storage temperature. This could be due to the decreased dynamic viscosity and enhanced diffusion constant at higher temperature.

There is another interesting work by Pongpeerapat et al. investigating probucol/PVP/SDS ternary ground mixture (GM) that was prepared with a vibrational rod mill [92]. The produced primary probutcol nanoparticles were around 20 nm in presence of both SDS and PVP. An interesting phenomenon was observed following the dispersion of the GM into water. For GM stabilized with PVP K17 and SDS, spherical agglomerates of primary nanocrystals were formed immediately in the size of around 90 nm after dispersion of the GM into water. A further agglomeration to around 160 nm in size occurred gradually during the storage stability study. In the case of PVP K12 and SDS, agglomerations of approximately 180 nm were observed after 4 days of storage and then remained stable up to 84 days. This phenomenon is illustrated in Fig. 5. Above critical aggregation concentration, SDS complexes with PVP to form a "necklace" structure in aqueous medium through both electrostatic and hydrophobic interactions. Following dispersion of probucol/PVP K17/SDS into water, PVP K17/SDS "necklace" complex interacted with primary drug nanoparticles, causing immediate agglomeration of the primary nanoparticles into 90 nm aggregates. The 160 nm secondary nanoparticles were formed due to further gradual agglomeration process. The stabilization of probucol nanocrystals was attributed to formation of PVP K17/SDS layered structure on the surface of probucol. For the GM of probucol/PVP K12/SDS, agglomeration of primary drug nanoparticles occurred more rapidly because of the insufficient surface coverage of PVP K12 and SDS on the probucol surface. Stabilization of the nanosuspension was linked to absorption of PVP K12 on the surface of probucol nanocrystals, owing to the absence of layered structure.

Despite the proven importance of stabilizers in preventing particle agglomeration, there have been a few studies that generated stable nanosuspensions without stabilizers. Baba et al. prepared 2-devinyl-2-(1-hexyloxyethyl)pyropheophorbide (HPPH) nanosuspensions without any stabilizer and reported formulation stability for more than 3 months [103]. The self-stabilization of the nanosuspensions was attributed to a high ZP value (−40 mv) resulting from the deprotonation of the carboxylic end group of HPPH molecules. A similar self-stabilized nanosuspension was reported in another study in which amorphous all-trans retinoic acid nanoparticles were shown to be stable in aqueous medium up to 6 months. Epstein et al. [95] prepared self-suspended alendronate nanosuspensions by combining the negative charged alendromnic acid with gallium (Ga) or

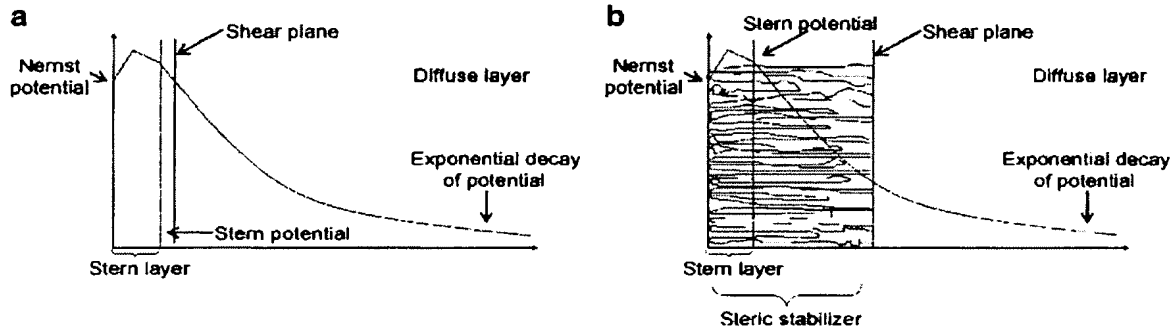

Fig. 4. Location of shear plane in an electrostatic stabilized system (a) and in a combined steric-electrostatic stabilized system (b). Reprinted from Ref. [30] with permission from ELSEVIER.

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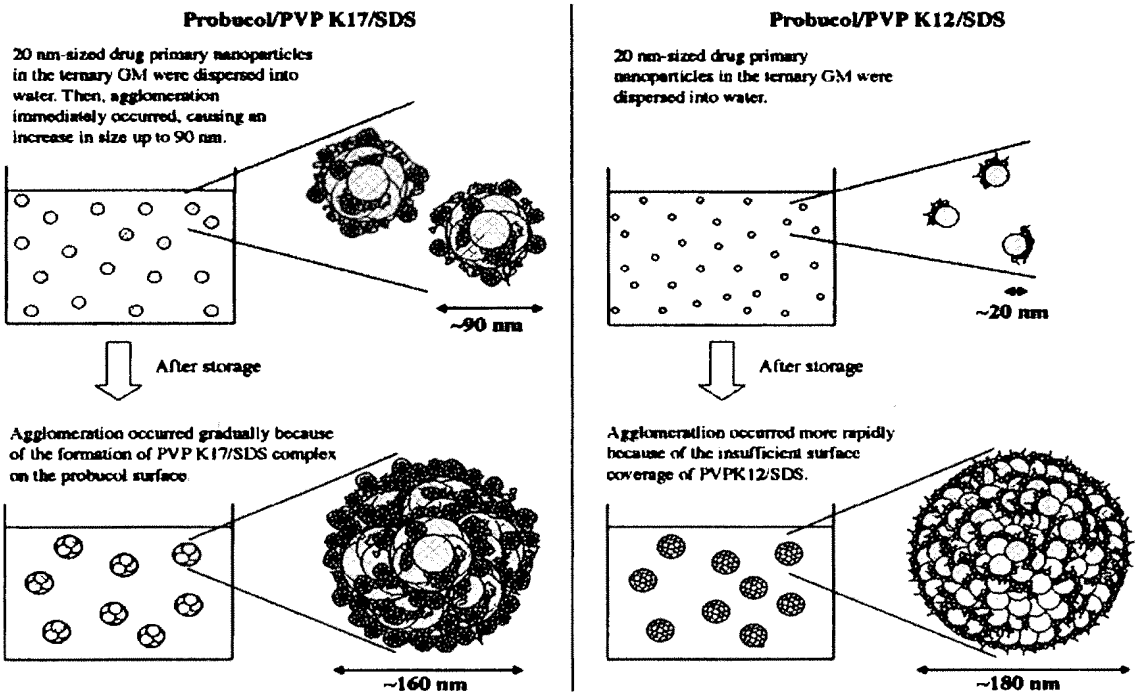

Fig. 5. Schematic overview of agglomeration/stabilization mechanism of probucol/PVP/SDS ternary ground mixture after dispersion into water. Reprinted from Ref. [92] with permission from ELSEVIER.

gadolinium (Gd) under sonication as complex nanoparticles. The alendronate-Ga nanosuspension was shown to be stable for more than 3 months, while the alendronate-Gd nanosuspension was stable for only 3 days. These stability profiles correlated well with their ZP values (33 mV for Ga complex vs. 21 mV for Gd complex).

2.2.3. Crystal growth

Crystal growth in colloidal suspensions is generally known as Ostwald ripening and is responsible for changes in particle size and size distribution. Oswald ripening is originated from particles solubility dependence on their size. Small particles have higher saturation solubility than larger ones according to Ostwald–Freundlich equation [115], creating a drug concentration gradient between the small and large particles. As a consequence, molecules diffuse from the higher concentration surrounding small particles to areas around larger particles with lower drug concentration. This generates supersaturated solution around the large particles, leading to drug crystallization onto the large particles. This diffusion process leaves an unsaturated solution surrounding the small particles, causing dissolution of the drug molecules from the small particles into the bulk medium. This diffusion process continues until all the small particles are dissolved. The Ostwald ripening is essentially a process where large particles grow at the expense of smaller particles [36,37], which subsequently leads to a shift in the particle size and size distribution of the colloidal suspension to a higher range. The diffusion and crystal growth during Ostwald ripening is shown schematically in Fig. 6.

A narrow particle size distribution can minimize the saturation solubility difference and drug concentration gradients within the medium, and thus help to inhibit occurrence of the Ostwald ripening [37]. This can perhaps explain why Ostwald ripening is not a major concern for nanosuspensions with uniform particle size [10,20]. Stabilizers may also alleviate Ostwald ripening as long as they do not enhance the drug solubility [116,117]. Being absorbed on the nanoparticles surface, the stabilizers can reduce the interfacial tension between the solid particles and liquid medium, and thus preventing the Ostwald ripening. Solubility, temperature, and mechanical agitation also affect Ostwald ripening [117]. Verma et al. produced ibuprofen nanosuspensions by microfluidization milling with the aid of various stabilizers (HPMC, Pluronic® F68 & F127, Kollidon 30, SLS) [31]. The particle size obtained with microfluidization showed some correlation with the ibuprofen solubility in aqueous stabilizer solutions. A higher solubility of ibuprofen in the solution of SLS, Tween 80 and Pluronic® F127 resulted in larger particles due to Ostwald ripening that occurred during process. A similar correlation was observed for ibuprofen particles during storage where Ostwald ripening was also believed to the driving factor for formation of larger particles. Van Eerdenbrugh et al. demonstrated that Ostwald ripening was highly dependent on temperature by exploring TPGS stabilized nanosuspensions for 9 different drug candidates [86]. Following 3 months storage at room temperature, Ostwald ripening occurred in 8 out of 9 nanosuspensions studied. Enhanced Ostwald ripening was observed at 40 °C storage, while lowering temperature to 4 °C slowed down or even stopped Ostwald ripening effects.

2.2.4. Change of crystalline state

Crystalline state is one of the most important parameters affecting drug stability, solubility, dissolution and efficacy. The main issue with crystalline state change is the transformation between amorphous and crystalline state. The high energy top-down manufacturing techniques tend to create partially amorphous nanosuspensions and some bottom-up techniques can create completely amorphous particles. The high energy amorphous particles are unstable and inclined to convert to low energy crystalline state over time. This conversion occurs depending on different parameters, such as temperature, dispersion medium, stabilizers and the presence of crystalline particles. Lindfors et al. produced Felodipine amorphous

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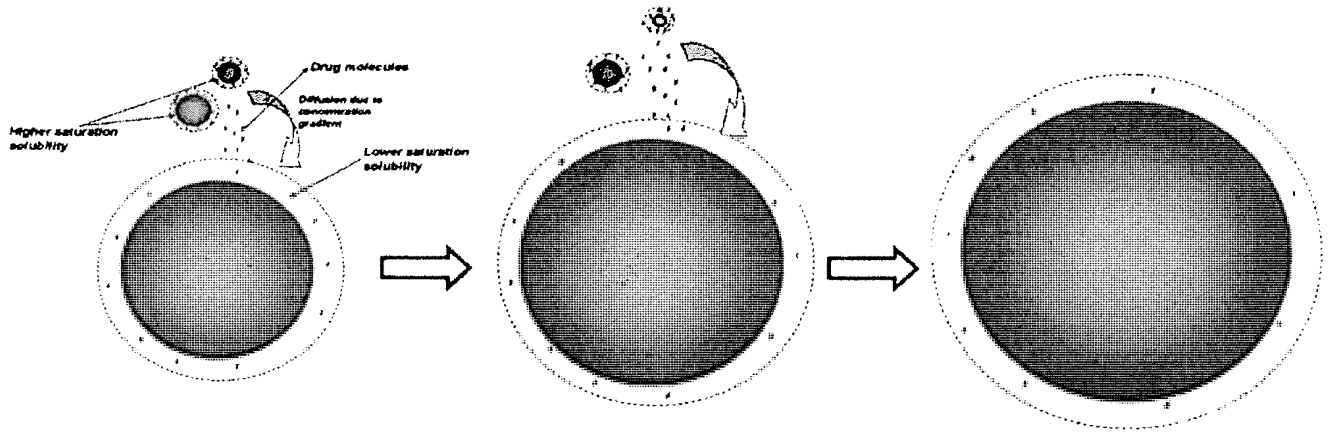

Fig. 6. Schematic illustration of Ostwald ripening.

nanosuspensions via anti-solvent precipitation under sonication [96]. They demonstrated that amorphous nanoparticles were highly unstable in the presence of small amounts of crystalline particles. This was attributed to saturation solubility differences between amorphous and crystalline nanoparticles that initiated a similar diffusion process to Ostwald ripening, leading to a rapid conversion of amorphous nanoparticles to crystalline state. Although most of amorphous particles have been shown to be unstable, a few amorphous nanosuspensions have been demonstrated to be stable over a certain period of time. Amorphous hydrocortisone nanosuspensions, produced through a bottom-up nanoprecipitation technique using microfluidic reactors, was found to remain stable after 3 months storage at room temperature [93]. Amorphous all-trans retinoic acid nanosuspensions, prepared by an anti-solvent precipitation technique, were also shown to be stable over 6 months storage at 4 °C [102].

Manufacturing process might also induce some other type of crystalline transformation. Lai et al. prepared the diclofenac acid (DCF) nanosuspensions by HPH with two different crystalline forms (DCF1 and DCF2) [60]. 5 w/w% Pluronic® F68 was used as a stabilizer. XRD analysis showed that these two crystalline forms belonged to the same polymorph with differences in molecular conformation and crystal size. It was demonstrated that the HPH process caused the partial transformation of DCF2 to DCF1 while no effect on DCF1 was observed. The change in the crystalline structure was attributed to the solubilization of DCF2 during HPH process and its subsequent recrystallization as the DCF1 form.

2.2.5. Stability issues with solidification process of nanosuspensions

When stable nanosuspensions are unattainable, the solid dosage form is the ultimate solution. The most common solidification processes are freeze drying and spray drying [10,19,20,118]. Since most solidified nanoparticle dry powders are usually reconstituted back into nanosuspensions during administration, drug nanocrystal growth or agglomeration during drying process needs to be prevented in order to maintain the nanosizing features such as rapid dissolution following the reconstitution. Adding matrix formers, such as mannitol, sucrose and cellulose, into nanosuspensions prior to drying is the common approach to overcome the stability issues during solidification process [19]. Since several excellent reviews have been published on this topic [19,25,118], the readers are directed to those reviews for more details.

2.2.6. Chemical stability

Since drug nanocrystals are usually dispersed in nanosuspensions with a limited solubility, the possibility of chemical reactions is not as substantial as that in solution-based formulations. Consequently, chemical stability of nanosuspensions is generally superior to that of solutions. Paclitaxel serves as a good example to illustrate this [119]. Fig. 7(a) shows an HPLC diagram of paclitaxel nanosuspensions stabilized with Pluronic® F68 after 4 years of storage at 4–8 °C. No visible degradation product was observed with a recovery of more than 99%. On the other hand, paclitaxel solution with methanol as cosolvent showed clear degradation only after 48 h at room temperature (Fig. 7(b)). The excellent chemical stability of paclitaxel nanosuspensions was attributed to a mechanism similar to oxidized layer on the aluminum surface. Monolayer degradation on the nanocrystals surface was created once they were exposed to water and oxygen, as illustrated in Fig. 7(c). This monolayer could protect the inner part of drug crystals from further degradation, and thus enhance chemical stability of the nanosuspensions.

Unlike the physical stability issue that is a common concern for nanosuspensions, chemical stability is drug specific. Each molecule has its particular functional groups and reaction mechanism that affects the stability. For example, chemical functionalities, such as ester and amides, are susceptible to hydrolytic degradation, while amino groups may undergo oxidative degradation [120]. Although chemical stability of nanosuspensions is usually not a major concern, extra attention should be paid to drug molecules with solubility greater than 1 mg/mL or with low concentration in suspension [120]. The common strategy to enhance the chemical stability is to transform the nanosupensions into dry solid dosage form which is much more stable than nanosuspensions [19] or to increase the concentration of the nanosuspensions [120].

2.3. Additional stability issues relate to large biomolecules

Large biomolecules discussed in this review are mainly referred to therapeutic protein and peptide. The molecular structure of protein/peptide is distinctly different and more complicated as compared to that of the small molecules. The structures of large molecules are generally differentiated into four structures, i.e. primary, secondary, tertiary and quaternary structures [34]. These different structures refer to the sequence of the different amino acids, regions where the chains are organized into regular local structures by hydrogen bonding such as alpha helix and beta sheet, the mechanisms on how the protein/peptide chain folds into a 3-dimensional conformation, and the composition of multiple protein/peptide molecules assembly, respectively [32,33,123]. The intact molecular structure of protein/peptide is essential to maintain their therapeutic efficacy [35,121]. Common stability issues associated with protein/peptide include deamidation, oxidation, acylation, unfolding, aggregation and adsorption to surfaces [35,121]. These stability issues are affected by

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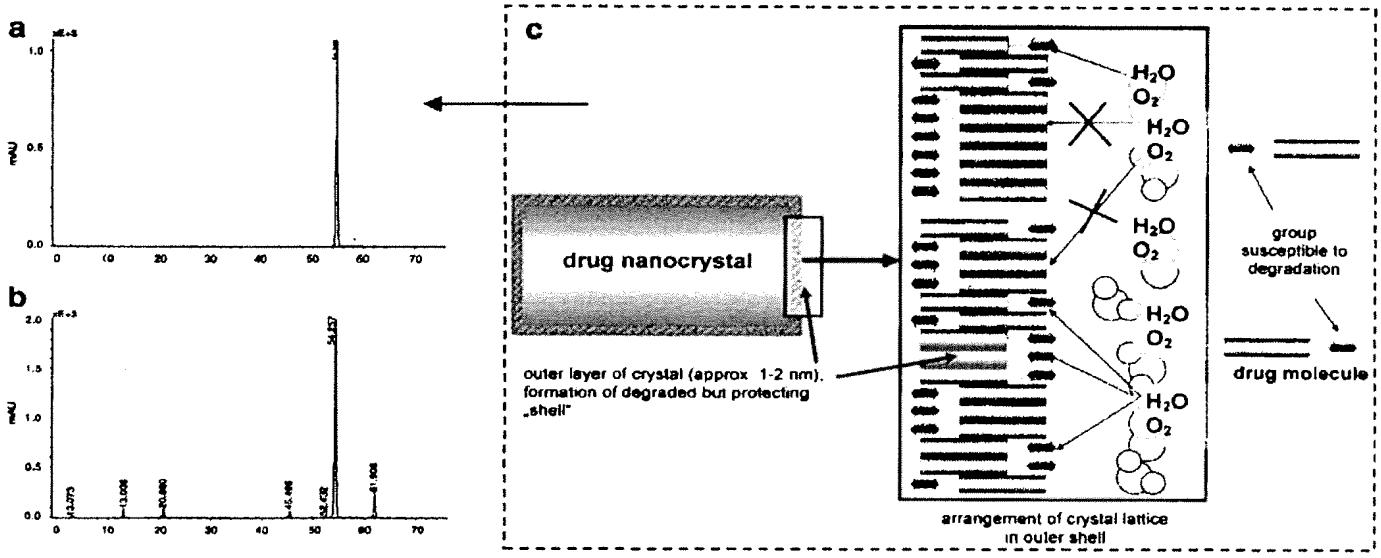

Fig. 7. (a) HPLC diagram of paclitaxel aqueous nanosuspensions stabilized with Pluronic® F68; (b) HPLC diagram of paclitaxel solution (methanol: 10 ml, water: 5 ml, paclitaxel: 20.8 mg); (c) Schematic illustration of stabilization mechanism of paclitaxel nanosuspensions. Reprinted from Ref. [119] with permission from ELSEVIER.

temperature, solution pH, buffer ion, salt concentration, protein concentration, and added surfactants, with solution formulations being more susceptible to the influence from these factors than the suspension formulations [34,35,121]. Although suspension formulations or solid state of protein/peptide have enhanced stability due to their reduced molecular mobility, other stability issues may arise during particle formation or formulation process. For example, irreversible denaturation and aggregation upon reconstitution were often observed for dehydrated protein through freeze drying or spray-drying [125,126]. To prevent this, supplementary excipients such as bulking agents or surfactants are usually introduced during lyophilization [122].

The vulnerable structure of protein/peptide creates challenges for formulation development. Instead of using "naked" protein, the common strategy to prevent protein/peptide denaturation is to encapsulate the biomolecules with carrier such as liposome [123], SLN [124] or polymeric materials [125,126]. In addition to improving the stability, protein/peptide encapsulation can enhance bioavailability and provide sustained therapeutic release [125–128]. There has been plenty of work reported on encapsulated protein/peptide nanoparticles but very scarce studies on pure protein/peptide nanoparticles. Gomez et al. produced bovine zinc insulin nanoparticles using an electrospray drying technique and reported retained biological activities of the particles [129]. By using HPH, Maschke et al. attempted to micronize insulin in the medium of Myglyol 812 [130]. The stability and bioactivity of the insulin were maintained in spite of the harsh HPH process conditions. Merisko-Liversidge et al. [83] also noticed retained stability and bioactivity of zinc-insulin nanosuspensions that were produced through a wet milling process in presence of Pluronic® F68 and sodium deoxycholate. Nyambura et al. utilized a bottom up technique (combination of emulsification and freeze drying) to generate insulin nanoparticles (80 w/w% insulin with 20 wt.% lactose) [110]. The particles were then dispersed into HFA134a to produce an MDI formulation. The molecular integrity of insulin formulation, measured by HPLC, size exclusion chromatography, circular dichroism and fluorescence spectroscopy, indicated that native structures (primary, secondary and tertiary) were retained after particle formation and formulation process. The presence of surfactant (lecithin) and lyoprotectant (lactose) was believed to be responsible for preservation of the insulin structures. In their follow up work [109], they applied a similar approach to produce composite nanoparticles of lysozyme and lactose for MDI formulations. The retained biological activity of lysozyme was enhanced with increasing lactose concentration in the particles, and reached maximum (99% retained activity) with 20 w/w% lactose. Nanoprecipitation coupled with freeze drying was used as well in this work to produce spherical nanoparticles containing 80 w/w% lysozyme with fully preserved bioactivity. It was demonstrated that bioactivity of lysozyme nanoparticles remained unchanged when in contact with HFA 134a. Yu et al. compared the effectiveness of spray freezing into liquid (SFL) and spray-freeze drying (SFD) processes in producing bioactive lysozyme particles [131]. Both processes generated highly porous micro-sized aggregates of lysozyme primary nanoparticles in the size of 100–300 nm. SFL process was shown to produce lysozyme with lower aggregation and higher enzyme activity as compared to the SFD process, which was attributed to the shorter exposure time to the air–water interface during the SFL atomization process.

3. Characterizing stability of drug nanoparticles and nanoparticle formulations

Selection of characterization techniques for drug nanoparticles stability is dependent on the nature of stability issues and product dosage form. A few commonly used stability characterization techniques are listed in Table 2.

3.1. Particle size, size distribution and morphology

Particle size and size distribution are the key parameters used for evaluating the physical stability of nanoparticles. A variety of techniques, including photon correlation spectroscopy (PCS), also known as dynamic light scattering (DLS), laser diffraction (LD) and coulter counter, are commonly used to measure the particle size and size distribution (Table 2). The PCS/DLS is widely used to determine the size and size distribution of small particles suspended in liquid medium. The mean particle size and size distribution indicated as polydispersity index (PDI) are the typical measured parameters of this technique. A PDI value of 0.1 to 0.25 indicates a narrow size distribution while a PDI greater than 0.5 refers to a broad distribution [20]. Unfortunately, this technique is not capable of measuring size of dry powders and its measurement range is too narrow (3 nm to 3 μm) to detect the interference from the microparticles (>3 μm) within the

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Table 2
Commonly used technique to evaluate the stability of nanoparticles.

Measured parameters	Techniques	Remarks
Particle size and size distribution	PCS/DLS	Pros: rapid, non-invasive. Cons: limited measurement range; apply only to liquid suspension.
	Laser diffraction	Pros: wide measurement range, rapid, non-invasive, apply to both liquid suspension and dry powder samples. Cons: particles are assumed to be spherical.
	Coulter counter	Pros: precise. Cons: apply only to spherical particles.
Particle size and morphology	SEM/TEM	Pros: evaluate both particle morphology and size, very small quantity of sample required. Cons: challenging to acquire statistical size distribution, usually invasive, time-consuming.
	AFM	Pros: non-invasive, evaluate both particle morphology and size, very small quantity of sample required. Cons: challenging to acquire statistical size distribution, time-consuming.
Sedimentation/creaming	Visual observation/laser backscattering/ near infrared transmission	-
Particle surface charge/zeta potential	Laser Doppler electrophoresis	-
Crystallinity state	XRD/DSC	-
Chemical stability	HPLC/FTIR/NMR/MS	-

nanosuspensions. Therefore, LD is often used in combination with PCS to circumvent this issue. Laser diffraction has a much wider detection range (20 nm to 2000 μm) and it can be used to evaluate both suspension and dry powder samples. The typical LD characterization parameters are LD50, LD90 and LD99, indicating 50, 90 or 99% of the particles are below the given size, respectively. LD is especially suitable for characterizing parenteral and pulmonary suspensions due to it wide measurement range. LD can detect the presence of microparticles (>5 μm) which are detrimental to parenteral nanosuspensions. However, LD provides only relative size distribution. The Coulter counter, on the other hand, measures the absolute number of particles per volume unit for the different size classes, and is more precise than the LD.

Although PCS, LD and coulter counter techniques provide rapid measurement of particle size and size distribution, they do not have the capability in evaluating particle morphology. As direct visualization techniques, Scanning Electron Microscope (SEM), Transmission Electron microscope (TEM) and Atomic Force Microscope (AFM) are widely used for assessment of particle morphology. However, it is very challenging and time-consuming to measure a significant number of particles to achieve statistical size distribution using these techniques. In addition, they usually require additional sample preparation such as coating that could be invasive to the particles, potentially causing some changes in particle properties.

3.2. Sedimentation/creaming

The traditional method to evaluate sedimentation or creaming is by visual observation over a period of time. By measuring the volume of the settled or creamed particle layer relative to the total suspension volume within a specific time, a dimensionless parameter known as sedimentation or flocculation volume can be obtained as a quantitative evaluation of suspension stability. A higher flocculation volume indicates a more stable suspension. The structure of settled/creamed layer can be easily assessed by re-dispersing the suspension, i.e. easily re-dispersed suspension indicates loose flocs while a dense cake is hard to be broken by manual shaking. Other approaches to evaluate sedimentation/creaming include laser backscattering [132] and near-infrared transmission [133].

3.3. Particle surface charge

Laser Doppler electrophoresis is commonly used to measure ZP. This technique evaluates electrophoretic mobility of suspended particles in the medium. It is a general rule of thumb that an absolute value of ZP above 60 mV yields excellent stability, while 30, 20 and less than 5 mV generally results in good stability, acceptable short-term stability and fast particle aggregation, respectively [29]. This rule of thumb is only valid for pure electrostatic stabilization or in combination with low-molecular weight surfactants, and is not valid when higher molecular weight stabilizers are present [29].

3.4. Crystalline state

The crystallinity of drug nanoparticles is usually assessed by X-Ray Diffraction (XRD) and/or Differential Scanning Calorimetry (DSC). XRD differentiates amorphous and crystalline nanoparticles as well as different polymorphic phases of the particles, while DSC is often used as a supplementary tool to XRD. Crystalline particles usually have a sharp melting peak which is absent in amorphous materials. The melting point can also be utilized to differentiate different polymorphs.

3.5. Chemical stability

HPLC is the most common characterization technique used to evaluate chemical stability that provides precise quantitative analysis on the degradation impurities. Mass spectrometry (MS) is often coupled with HPLC to identify the molecular structure of impurities. Some other techniques such as FTIR and NMR can also be used for chemical stability assessment. However, they are not as precise and sensitive as HPLC, and thus not widely used for stability assessment.

3.6. Additional techniques for assessing large biomolecule nanoparticle and formulation stability

For large biomolecules, additional characterization tools are generally required depending on the level of molecular structure to be assessed. For instance, size exclusion chromatography and electrophoresis are used to evaluate the primary structure of large biomolecules, circular dichroism is to monitor the secondary and tertiary structures while fluorescence spectroscopy is for tertiary structure [34,134]. In addition, *in-vitro* bioassays or *in-vivo* efficacy tests are needed to evaluate biological activities of the large biomolecules. Insulin particles, as an example, have been tested for its bioactivity either by *in-vitro* chondrocyte culture assays [130] or *in-vivo* monitoring of blood glucose level on rats following insulin administration [83].

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4. Recommendations of general strategies for enhancing stability of nanoparticle formulations

Strategies to address different stability issues are usually tailored according to different aspects, such as therapeutic requirements, dosage form and manufacturing complexity. For example, as the particle size is reduced, the sedimentation rate is decreased so that the particles can stay suspended longer in nanosuspensions. The general wisdom is that the smaller the nanoparticles are, the better. Unfortunately, too small particles are not always desirable, as they may create undesired plasma peaks due to the significant increases in dissolution rate [28]. Moreover, manufacturing complexity may be increased as well when the particles size requirements become too stringent.

The use of stabilizers is the most commonly used technique in achieving a stable nanoparticle formulation. However, the stabilizer selection is known to be very challenging. The challenge stems mainly from two aspects: (i) lack of fundamental understanding of interactions within nanosuspensions and (ii) lack of an efficient and high throughput stabilizer screening technique. In the case of aqueous nanosuspensions, it is relatively easy to select stabilizers given that water-based stabilizing moieties such as PEG and PVA are well known. However, selecting the anchor groups that interact strongly with the drug surface can be challenging due to the limited understanding on interactions between nanoparticles and stabilizers in molecular level. For non-aqueous nanosuspensions such as HFA-based MDI delivery system, understanding of solvation in the low-dielectric HFA medium is still in its infancy, which makes stabilizers selection even more challenging. Inefficient screening approaches are another hurdle for stabilizer selection. The current practice for stabilizer screening involves trial production of nanosuspensions with different stabilizers or stabilizer combinations, which could be burdensome and require vast amount of efforts especially with a large number of potential stabilizer candidates. AFM has recently been proven to be a feasible and efficient tool for stabilizer screening. Verma et al. demonstrated the feasibility of using AFM to select stabilizers for Ibuprofen nanosuspensions [135]. The AFM measurements showed that HPMC and HPC had extensive surface absorption on the ibuprofen surface, as opposed to the inadequate surface absorption with PVP and Pluronic® surfactants. These results correlated well with their stabilizing performances in the nanosuspensions. This finding confirmed the significance of AFM in providing a scientific rationale for stabilizer selection and improving understanding of the stabilization mechanisms. Another technique, known as colloidal probe microscopy (CPM) which is derived from AFM, has also been widely used to study interactions between colloidal particles and is expected to be a useful tool for nanosuspension stabilizer screening [136].

Due to the significant challenges associated with stabilizer selection, self-stabilized nanosuspensions with no added stabilizer are highly desirable. This is not only for simplifying the formulation development process but also reducing stabilizer-based toxicity. Unfortunately, the challenges to engineer such self-suspended nanoparticles are tremendous with very few reported studies to date. A couple of approaches that could potentially be used to produce self-stabilized nanosuspensions include the creation of drug nanoparticles with high ZP and controlling morphology or surface properties of drug nanoparticles to minimize inter-particulate forces.

5. Conclusions

The stability of drug nanoparticles remains a very challenging issue during pharmaceutical product development. Stability is affected by various factors such as dosage form (nanosuspension vs. dry solid), dispersion medium (aqueous vs. non-aqueous), delivery route (oral, inhalation, IV or other routes), production technique (top-down vs. bottom-up) and nature of drug (small molecules vs. large biomolecules). Despite the significant challenges associated with stabilizer screening, adding a stabilizer or combination of stabilizers is still the most commonly used and preferred approach to enhance the stability of nanosuspensions. Further understanding of particle–particle interactions within nanosuspensions and development of high-throughput stabilizer screening tools are essential to facilitate efficient stabilizer selection. Development of self-stabilized nanosuspensions, although currently seen as very complicated and challenging, is expected to grow with the continuing advancement in the field of particle engineering.

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